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To my family

ABSTRACT

Four equine adenovirus isolates were characterized with regard to their immunological, biophysical and biochemical properties.

Primary cell cultures, derived from fetal equine kidney and feline kidney, were found to be highly susceptible to equine adenovirus infection, with equine cells being the system of choice for viral purification and quantitation. Concentration and purification of the four virus isolates was most efficiently accomplished by a combination of freeze-thawing and sodium deoxycholate extraction, followed by two cycles of CsCl gradient centrifugation.

Plaque morphology and size was similar for all four virus isolates when assayed in primary equine fetal kidney cells. Examination of thin-sections of virus-infected cells revealed that equine adenovirus multiplication and assembly occurred within the cell nucleus. The one-step growth cycle of a representative equine adenovirus isolate was found to be similar to that of the human adenoviruses, but with a shorter time course consisting of a 16 h latent and a 16 h rise period. Mature virus particles were found to be mainly cell-associated.

Cross serum neutralization and hemagglutination-inhibition assays, performed with purified equine adenovirus isolates and specific antisera, revealed no distinct antigenic diversity among the four isolates. The antigenic relationship of the isolates was further confirmed by neutralization-enhancement assay, which can detect minor shared or distinct antigenic sites on the virus capsid. Low levels of hemagglutinating activity associated with oligomeric structures (penton or fiber dimers) were found in the pool of equine adenovirus soluble

components. Hexon-associated group specific activity was the only inter-species specificity detected in association with the equine adenovirus isolates.

Ion-exchange chromatography indicated the presence of the same relative net charge on the hexon component of all four equine adenovirus isolates. The buoyant density in CsCl of purified virions was found to be 1.344 to 1.345 g/ml, and the virion of each isolate was found to be most stable in acidic pH and 3 M CsCl at room temperature or 4° C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified virions of the four equine adenovirus isolates revealed the presence of 12 to 14 polypeptides of molecular weight ranging from 9,500 to 145,000. The electrophoretic profile and calculated molecular weight of each polypeptide was found to be similar for all four isolates.

The morphology of the equine adenovirus virions, as revealed by electron microscopy of negatively-stained preparations, was found to be that typical for the adenovirus group, ie., cubic (icosahedral) symmetry with a fiber projection at each vertex of the virion. The 50 nm fibers observed in association with intact virions are the longest thus far reported for members of the adenovirus group. The equine adenovirus genome was observed to be a linear duplex molecule devoid of apparent segmentation or circular structure. The molecular weight of the observed genome, as determined by electron microscopic measurement, was calculated to be 21-22 x 10^6.

This detailed study of four equine adenovirus isolates has confirmed that the isolates most probably represent a single antigenic serotype.

"What is new and significant must always be connected with old roots, the truly vital roots that are chosen with great care from the ones that merely survive."

Bela Bartok

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ABBREVIATIONS

CIE	counterimmunoelectrophoresis
CPE	cytopathic effect
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
EAdV	equine adenovirus virion
EDC	equine dermis cells
EDTA	(ethylenedinitrilo) tetraacetic acid, disodium salt
EPK	equine primary kidney
FPK	feline primary kidney
g	gravity (centrifugal force)
HA	Hemagglutination
h	hour
HAU	hemagglutination unit
HFS	human foreskin
K	1000 daltons
M	molar
MDCK	Madin Darby canine kidney
mM	millimolar
MEM	minimum essential medium
min	minute
MOI	multiplicity of infection
µg	microgram
µl	microliter
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PFU	plaque forming unit
PI	post-infection
PBS	phosphate buffer saline
RPK	rabbit primary kidney
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
SSTA	sodium silicotungstate acid
Tris	tris (hydroxymethyl)-aminomethane
$TCID_{50}$	tissue culture infective dose
w/v	weight per volume

INTRODUCTION

The term adenovirus was first used in 1956 (Enders et al.) to designate a group of viruses isolated from the respiratory tract of man and other animals. Adenoviruses can cause primarily mild respiratory disease and, less frequently, conjunctivitis, myocarditis, and lymph node involvement (Sohier et al., 1965). Adenoviruses are non-enveloped, icosohedral entities with a double-stranded DNA genome larger than that of the papova-viruses, but smaller than that of the pox-viruses, T-even bacteriophage and the herpes viruses. The genome has a MW about 20-25 x 10^6 and therefore can code for 25-50 average size polypeptides. Thus far, close to one hundred different serotypes have been isolated from a variety of species (Norrby et al., 1976). All adenoviruses except those of avian origin seem to share at least one antigenic determinant. It has been proposed that the family of "Adenoviridae" should be divided into two genera named "Mastadenovirus" and "Aviadenovirus" (Norrby et al., 1976). The main reason for the segregation is the absence of any immunological cross-reacting structural protein in avian and mammalian adenoviruses (Kawamura et al., 1965; Maferran et al., 1972). In addition, it has also been reported that avian adenoviruses contain a relatively larger amount of DNA, but fewer polypeptides than mammalian adenoviruses (Laver et al., 1971). Different adenovirus serotypes also possess a high degree of individuality, such as cytopathology, hemaglutination properties, neutralization kinetics, oncogenic potential and host range.

The human adenoviruses have been classified into subgroups on the basis of their ability of agglutinate rhesus monkey *(Cercopithecus aethiops)* erythrocytes (Rosen, 1958) and also on the basis of their oncogenicity in rodent neonates (Huebner, 1967). Each subgroup contains several serotypes which can be characterized by type-specific antigens present in their capsids as revealed by hemagglutination inhibition and neutralization tests.

Adenoviruses have received much attention during the last fifteen years because of their oncogenic potential in neonatal rodents. It was discovered that human adenovirus type 12 (HAd-12) induced tumors in newborn hamsters (Trentin et al., 1962). The same property was reported for HAd-18 by Huebner et al. (1962). The oncogenicity of HAd-12 in rodent neonates, and also of several other human and non-human adenoviruses has been confirmed in many laboratories (Huebner et al., 1963; Rabinson et al., 1964; Yabe et al., 1964; Pereira et al., 1965; Huebner et al., 1965; Girardi et al., 1964; Hull et al., 1965; Darbyshire, 1966; Sarma et al., 1965). Adenovirus-induced tumors are usually undifferentiated sarcomas, although malignant lymphomas have been observed (Larson et al., 1965).

Cellular transformation is another feature associated with certain adenoviruses and was first demonstrated by Macbride & Weiner (1964). It was shown that HAd-12 could transform *in vitro* cultures of newborn hamster kidney cells. Freeman et al., (1967) have also reported the transformation of rat embryo fibroblasts by HAd-12.

Conditional-lethal (temperature-sensitive) mutants represent another interesting area in the study of adenoviruses. A large number of ts-mutants of HAd-2, HAd-5, HAd-12 and CELO, (Avian adenovirus;

cytopathic enteric lethal orphan virus) viruses have been described (Williams et al., 1971; Lundholm & Doerfler, 1971; Ensinger & Ginsberg, 1972; Shiroki et al., 1972; Ishibashi, 1970, 1971). More than fifty HAd-5 ts-mutants have been isolated and extensive biochemical and genetic analyses are currently underway. During recent years, new and sensitive physico-chemical techniques allowing the mapping of loci for specific functions on viral chromosomes have been developed. Adenoviruses have also become a very powerful tool in providing a model system for studies of the regulation of gene expression in eukaryotic cells (Philipson et al., 1975). *In vitro* biochemical studies of adenovirus-transformed cells have added much to the basic knowledge concerning growth regulation and tumorigenesis (Ponten, 1971).

Structure of the Adenovirus Particle

The architecture of the adenovirus particle (HAd-5) was first described by Horne et al. (1959). The 65-80 nm capsid is composed of 252 capsomers, arranged in icosahedral symmetry with 20 triangles and 12 vertices. 240 of the 252 capsomers have six neighbours and are called hexons (Ginsberg et al., 1966) and the 12 capsomers at the vertices have five neighbours and are called pentons (Ginsberg et al., 1966). Each penton unit consists of a penton base, which is anchored in the capsid, and a fiber projection (Valentine & Pereira, 1965; Norrby, 1966). The fiber has a knob attached at the distal end. The percentage weight of the capsid components account for approximately 55-60% of the protein content of the virion.

The structural proteins of the adenoviruses correspond morphologically and immunologically with viral specific proteins found free

in infected cell extracts (Norrby, 1966). These proteins are soluble in the native state and are called "soluble components". The soluble components are synthesized in large excess in the cytoplasm and only a small percentage is transported to the nucleus (Bello & Ginsberg, 1969) where virions are assembled (Thomas & Green, 1966; Velicer & Ginsberg, 1968). These structural proteins can first be detected 2-4 h before the maturation of virions (Russell et al., 1967; Schlesinger, 1969). Under one step growth conditions, when detecting the structural proteins by complement fixation at 5-24 h PI, Philipson and Pettersson (1973) reported the order of appearance of these proteins as hexon then penton base and last, major core protein. All proteins synthesized in the cells at late stages of infection are coded for by viral DNA (Ginsberg et al., 1967).

An excess pool of soluble components accumulates in the infected cells and can be isolated and characterized by numerous techniques such as immunoelectrophoresis (Pereira et al., 1959), ion-exchange chromatography (Klemperer & Pereira, 1959; Philipson, 1960; Wilcox & Ginsberg, 1961) and sucrose gradient zonal centrifugation (Marusyk et al., 1970). Boulanger and Puvion (1973) devised a three step purification procedure consisting of neutral salt precipitation, ion-exchange and adsorption chromatography for the large scale purification of hexon, fiber and penton components.

Hexon Component

Electron microscopy of the hexon has revealed a complex structure with a diameter of 8-10 nm and a central hole 2.5 nm in diameter (Pettersson et al., 1967). Various methods, including

ultracentrifugation, exclusion chromatography and X-ray crystallography, have been used to determine the molecular weight of the hexon. The molecular weight appears to be in the range of 330,000 - 360,000 (Franklin et al., 1971). The polypeptide composition of the hexon was studied by Maizel et al., (1968) using a neutral, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis system. It has been shown that the hexon contains a single component polypeptide of 120,000 daltons, implying that the hexon consists of three identical subunits. Various X-ray crystallographic studies have also suggested that hexons contain several subunits in multiples of three (Cornick et al., 1971; Franklin et al., 1971). The amino acid composition of HAd-2, HAd-3 and HAd-5 hexon has been determined and no major differences have been found between hexons from different serotypes (Biserte et al., 1966; Pettersson et al., 1967; Boulanger et al., 1969; Laver, 1970; Pettersson, 1971). They are all rich in dicarboxylic amino acids, and no carbohydrate has been detected (Pettersson et al., 1967; Boulanger et al., 1969).

The biological function of the hexon components has been well characterized by different workers. A group-specific specificity "α" (Pereira et al., 1963) was first recognized in the adenovirus hexon and is known to occur in all adenoviruses except those of avian and possibly bovine origin. Cross-adsorption and immunodiffusion studies revealed an additional type-specific specificity in the hexon referred to as "ε" (Norrby, 1969). It has been shown that group-specificity is localized at the inside of the capsid and type-specificity at the surface of the capsid. A more complex arrangement of the antigenic determinants of the hexon was later suggested by Norrby (1969 a, b) and Norrby & Wadell

(1969) as type, group, intrasubgroup and intersubgroup specificities could be detected by cross-adsorption and complement-fixation studies. The role of the hexon as a surface antigen for inducing neutralizing antibody has been demonstrated, and it was suggested that neutralization tests be used for primary classification of adenoviruses into serotypes because of the individualistic behavior of each serotype in the test.

Penton Base Component

The penton base has been purified by techniques similar to those used for hexons. This component is labile and present in low concentration in infected cells and is not as well characterized as the hexon. Morphologically, the five-sided penton base is very similar to the hexon with a diameter of 8 nm and a central hole 2.5 nm in diameter (Petterson & Hoeglund, 1969). Aggregates of pentons (penton base + fiber) can form oligomeric structures such as dimers, and dodecons (symmetrically arranged aggregates of 12 pentons; Norrby, 1966) both of which act as complete haemagglutinins.

Neutral SDS polyacrylamide gel electrophoresis has indicated that the penton base is composed of a single polypeptide with a molecular weight of approximately 70,000. Sedimentation and analytical centrifugation analysis indicate a molecular weight of 400,000 - 500,000 for the penton base plus fiber complex. Pentons of all serotypes may be detected as incomplete hemagglutinins (Norrby, 1966; Norrby, 1968; Norrby & Skaeret, 1968; Norrby & Ankerst, 1969; Norrby et al., 1967; Wadell & Norrby, 1969; Wadell et al., 1969). Pentons can be separated by zonal centrifugation into monomeric and oligomeric forms and only the latter have been found to carry complete haemagglutinating (HA) activity

(Wadell et al., 1969). The penton incomplete HA can be demonstrated in hemagglutination-enhancement (HE) tests (the aggregation of monomeric pentons by using antibodies present in heterotypic antisera which can react with penton base, i.e. the antigen specificity "β"). In addition, the penton contains intersubgroup and intrasubgroup specificities as demonstrated by determination of the immunological characteristics of HE antibodies in adsorption experiments (Norrby, 1969; Wadell & Norrby, 1969b). No type-specific determinant has been found on the penton base by the use of such techniques. This does not, however, exclude the possible presence of a determinant of this kind.

Fiber Component

Each penton base has attached to it a projecting fiber terminating in a small knob, usually 4-6 nm in diameter. Differences have been observed with regard to the length of the fiber from different serotypes and such observed differences have been used for classification (Norrby, 1969b; Liem, 1971; Laver et al., 1971; Marusyk et al., 1970). The fiber can be purified using similar techniques to those used for purification of the other capsid components. It appears that the diameter of the fiber is constant at approximately 2 nm, but the length varies within the different species subgroups of adenoviruses. For serotypes with longer fibers a positive correlation has been found between length and antigenic complexity. Each fiber has two determinants, "γ" which reside in the knob and is responsible for type specificity and "δ", located at the proximal end and is responsible for intrasubgroup specificity. Antibody against the "γ" determinant prevents attachment of fibers to KB and HeLa cell receptors (Philipson et al., 1968).

Interaction of anti "γ" antibodies with virions cause aggregation of the virus particles (Prage et al., 1970). The "δ" determinant is probably located near the junction between the fiber and penton base since it cannot be detected in the intact penton structure (Pettersson & Hoeglund, 1969; Wadell & Norrby, 1969). The estimation of the molecular weight of HAd-2 fibers as 200,000 (Philipson & Pettersson 1973), and of fiber-polypeptides as 62,000 in SDS-PAGE systems (Maizel et al., 1968) suggests that fibers contain 2-3 identical subunits.

Core Component

A comparison of the amino acid composition of the virion and purified hexons suggested the presence of an arginine rich protein within the virion particle (Russell & Knight, 1967). One possible role for arginine residues within the core is that the residues may be evenly distributed along the polypeptide chain in such a way that half of the phosphate groups on the adenovirus DNA may be neutralized (Prage & Petterson, 1971). Purification of a major core protein has been achieved only with disrupted virions as starting material. The core proteins were extracted with dilute acids and non-denaturing preparative polyacrylamide electrophoresis at pH 4.6 was used for the final purification (Prage & Pettersson 1971). The molecular weight of the major core protein has been estimated to be 18,000 by equilibrium centrifugation (Prage & Pettersson 1971) and SDS polyacrylamide gel electrophoresis (Maizel, 1971; Everitt et al., 1973; Anderson et al., 1973). The major core proteins from HAd-2 and HAd-3, which belong to different subgroups, share some immunological properties. It has been

postulated that the major core protein is essentially a maturation factor (Rouse & Schlesinger, 1967; Russell & Beiker, 1968). Everitt et al. (1973) observed that the majority of the viral-specific proteins assemble into mature virus only when medium containing arginine is added to arginine-depleted infected cells, strongly suggesting that the synthesis of adenovirus particles is dependent upon the concentration of arginine in the growth media.

The presence of an endonuclease activity in association with human adenoviruses and excess pool pentons has been reported (Burlingham et al., 1971; Marusyk et al., 1975). The enzymatic activity was correlated with the finding of a slowly sedimenting (18S) viral DNA species in infected cells and in vitro incubation mixtures of intact viral DNA and HAd-2 and HAd-12 virions or excess pool pentons. The endonuclease activity was reported to make double-stranded scissions in substrate DNA. The endonuclease activity has been also detected in association with highly purified virions, pentons, and dodecons of adenovirus belonging to the three subgroups of human adenoviruses (Marusyk et al., 1975). The results of these experiments using a highly sensitive ethidium bromide fluorimetric assay have shown that the endonuclease activity is ubiquitous within the adenovirus group. Marusyk et al. (1975) were also able to demonstrate that the initial enzyme-like activity is manifested through a single strand scission of substrate DNA. The functional role of the enzyme-like activity is not yet understood.

Serotyping of Adenoviruses

Different methods have been described for typing adenoviruses. These methods include neutralization (Huebner et al., 1954) complement-fixation (Pereira, 1956), hemagglutination-inhibition (Rosen, 1960), immune-electron microscopy (Luton, 1973; Vassal & Ray, 1974), macrophage migration-inhibition (Novotny et al., 1974), counterimmunoelectrophoresis (Hierholzer & Brame, 1974; Nasz, 1967) and single radial-immunodiffusion (Grandien & Norrby, 1975). One of the important problems of typing adenoviruses is the adoption of criteria which firmly establishes such a classification. Taking advantage of the occurrence of a mosaic of multirelated structural components in adenovirus capsids was suggested by Norrby (1969). The primary question then would be which of the type specific components, hexon (ε) or fibers (γ), should be given priority? In practice one can choose between the use of the neutralization test or the hemagglutination-inhibition test with soluble components or virions. Acrording to the "International Committee of Nomenclature of Viruses (ICNV)" in Madrid, September , 1975, classification is based primarily on differences in immunological properties of the virions (Table 1).

Equine Adenoviruses

Adenoviral infection of the respiratory tract have been reported in a wide variety of domestic and wild animals: bovine Darbyshire and Pereira (1964), porcine (Bibrack, 1969) canine (Swango et al., 1970), avian (Burke et al., 1968), ovine (Macferren et al., 1971), and equine (Todd, 1969; McChesney et al., 1970). Other

Table 1

Adenoviruses Isolated from Different Species

Natural host	Serological types
Human	33 (35?)
Simian	24
Bovine	8
Canine	2
Equine	1
Murine	2
Ovine	3
Porcine	4
Fowl	9
Goose	3
Turkey	2
Frog	?

Modified from Norrby et al. (1976)

viral agents such as herpes viruses, myxoviruses, picornaviruses, reoviruses, equine arteritis virus, plummer equine respiratory virus ("PERV") and Kentucky equine respiratory virus ("KERV") have also been shown to cause respiratory infection in horses.

The equine adenoviruses,isolated in different areas of the world, are the least studied member of this group and have not been characterized to any extent. The equine adenoviruses are most frequently isolated from Arabian foals (Todd, 1969; McChesney, et al., 1970; Harden, 1974; Ardans et al., 1973; Dutta et al., 1975) and occasionally from thoroughbred foals (Petzolate & Schmidt (1971), Konoishi et al.,(1977). Wilkins & Studdart (1972) have also isolated equine adenovirus from a three day old Clydesdale horse.

The occurrence of equine adenovirus infection has been diagnosed on clinical, serological and histological grounds, Johnston & Hutchins (1967) McChesney et al., 1970; Darbyshire & Pereira, 1964; Afshar, 1969; Timoney, 1971; McGuire et al., 1973). In Australia, equine adenovirus infection was first diagnosed on the basis of histological evidence Johnston & Hutchins (1967). Distinctive pulmonary lesions with large intranuclear inclusions were observed and the authors concluded that because of the resemblance of observed inclusions to those previously observed in the livers of dogs with infectious canine hepatitis (ICH) and because the pulmonary lesions were comparable to those known to occur in adenoviral infections of other species, such as human , calves, swine, the infection should therefore be considered as an adenoviral bronchitis.

Clinical examination of victims of equine adenovirus infection generally reveals a poor physical condition with weakness, rapid tiring

alert appearance and normal reaction to external stimuli. Infrequently, a deep, soft productive cough with labial mucosa and mucopurulent nasal and ocular discharges are evident. Body temperature is usually high and the total white blood cell count is below normal range. B & T lymphocyte immunodeficiency in sibling Arabian foals has been shown (McGuire et al., 1973; McGuire et al., 1974). These foals were victims of equine adenovirus infection.

This class of immunodeficiency involves defects in both antibody production and cell-mediated immunity and is classified as a severe combined immunodeficiency. The defect in the B-lymphocyte system is manifested by hypophogammaglobulinemia, lymphopenia and the absence of germinal centers and the T-lymphocyte defect by an almost total absence of thymic tissue and lack of thymic dependent lymphocytes in the spleen of the victims. Discovery of this type of deficiency in Arab foals in association with equine adenovirus infection is another interesting finding associated with these viruses.

Sequential changes induced by an equine adenovirus in cultured fetal equine kidney cells were studied by electron microscopy (Shahrabadi, et al., 1977). Inclusions produced by the equine adenovirus were shown to differ from those produced by human adenoviruses. Some similarities were observed between inclusions produced by canine adenovirus and those produced by the equine adenovirus. Five different inclusion types were observed during the lytic cycle of equine adenovirus starting at 16 h PI and were designated type I to type V (48 h PI). The nature and chemical composition of the inclusions was determined by autoradiography and it was shown that types I, II, III were composed of nucleoprotein and type IV was composed of protein. The virus particles

accumulated in the central region of the type IV inclusion. The type IV inclusions showed a high degree of resemblance to the dark hexon-containing inclusions found in the nuclei of canine adenovirus-infected cells (Yamamoto & Shahrabadi, 1971). At 48 h PI some cells showed nuclear membrane disintegration which seemed to be the result of a mechanism other than mechanical breakdown. The possibility of enzyme activation for the purpose of virus release has been postulated for the equine adenoviruses, (Shahrabadi, et al 1977)

Before the course of this study only a brief survey of the serological relationship of different equine adenovirus isolates had been carried out (Studdart, 1974). No attempt has been made to purify the different capsid components of these viruses or to study the distinct serological relationship of different isolates in a sensitive serological system. Previous results from serological tests with crude material have shown that six adenoviruses isolated in the United States, Germany and Australia were closely related (Studdart et al., 1974). It has been previously shown, however, that the results of serological tests with crude material and antibody directed against crudely prepared virus are not conclusive (Marusyk, 1972; Stinski et al., 1975).

In this study an attempt has been made to undertake a complete systematic biophysical and serological study of four equine adenovirus isolates, as well as to elucidate the structure of this group of adenoviruses with regard to morphology and polypeptide composition.

MATERIALS AND METHODS

1. Virus Isolates

The four equine adenovirus (EAdV) isolates chosen for study were the kind gift of Dr. T.B. Crawford (Dept. of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, U.S.A.): EAdV-004 and EAdV-020 (originally isolated in Washington state), EAdV-Briarwood and EAdV-008 (originally isolated in Colorado). All isolate suspensions received were filtered through a 300 nm Millipore filter and propagated in equine primary kidney cells derived from 2 to 9-month-old equine fetal kidneys. Frozen stocks of virus isolates were stored at -35°C.

2. Cell Culture

Equine primary kidney cells were derived from equine fetal kidney. Equine fetuses were obtained from Alsask Processors (Division of Norfish Ltd.), Edmonton, Alberta. All fetuses were obtained between two to three hours after slaughter and carried immediately to the laboratory where nephrectomy was performed. Excised kidneys were immersed in 50 ml of PBS (free of Mg^{2+} and Ca^{2+}) containing 10^5 units of penicillin-G, 200 ug of streptomycin sulfate and 20 ug per ml gentamicin. Following 30 min incubation of the kidneys at 4° C, the fat tissue and capsules were removed aseptically and the remaining tissue was minced thoroughly and washed with several volumes of PBS. Three to four cycles of trypsinization at room temperature were

performed and the cells were centrifuged at 700 *g* for 15 min and seeded into 75 ml plastic tissue culture flasks. Passage one of the cells was trypsinized, the cell concentration adjusted to 1-2 x 10^6 cells per ml and the cells suspended in 2 ml aliquots in 10% DMSO and frozen in liquid nitrogen. Viability and sterility of each batch was examined after one week.

3. Cell Culture Medium

Minimum essential medium (MEM-Eagle, modified Auto-Pow, Flow Laboratories, Inc., Inglewood, Ca.) was dissolved in distilled water and autoclaved for 15 min. at 15 psi pressure, 120° C, and prior to use supplemented with 5% calf serum and 5% lactalbumin hydrolysate (Gibco Diagnostics Ltd., Calgary, Alta.).

Glutamine and 7.5% sodium bicarbonate were added to a final concentration of 0.002 M and 0.1% (w/v) respectively. A complete monolayer was obtained in three to four days after seeding. The cells were then:a)infected at 80% cell confluency with virus; b) incubated at 37° C until subculturing. Two percent calf serum plus 1.5 mM L-arginine were added to the maintenance medium and to each liter of medium, 10 ml of an antibiotic solution (10^5 units of penicillin, 200 ug of streptomycin per ml) was added before use.

4. Propagation of Virus

Eight to ten Blake bottles of equine primary kidney cells at passage levels of six or less were routinely used for virus propagation. Before infection of the monolayer, the tissue culture medium was

removed, and 5 ml of virus suspension at a multiplicity of infection of 5 $TCID_{50}$ per cell was added to each bottle and the virus was adsorbed, at 37° C for 60 min with the bottles being rotated at 20 min intervals. After the adsorption period, 90 ml of fresh cell culture maintenance medium was added and the infected cells were again incubated at 37° C until complete CPE was evident, usually in three days.

5. Purification and Concentration of Virus

Infected cells were scraped off from the glass using a rubber policeman and all cells were pooled and centrifuged at 700 *g* for 20 min. The supernatant was discarded and the cell pellet was suspended in 30 ml of 0.02 M Tris-Hcl, pH 9.0, and kept frozen at -35° C until used. For purification of virus:

a) the frozen pellet was suspended in 25 ml of 0.2% (w/v) sodium deoxycholate in 0.02 M Tris-Hcl, pH 9.0, and mixed by continuous shaking, at room temperature for 60 min. Cell debris was removed by one cycle of centrifugation at 800 *g* and the supernatant was layered on top of a preformed non-linear gradient of CsCl consisting of 5 ml of densities of 1.40 g/ml, 5 ml 1.32 g/ml and 5 ml of 1.20 g/ml, respectively, and centrifuged at 70,000 *g* for 90 min in a Beckman ultracentrifuge (Beckman Instruments, Palo Alto, Ca., U.S.A.), using a SW27 rotor (Marusyk et al., 1970; Marusyk, 1972). The region above the opalescent band (corresponding to equine adenovirus virion) was pooled and stored at -35° C as soluble virus components. The complete band of virus was removed, and the virus suspension was further purified by centrifugation to equilibrium in a CsCl solution, initial density of 1.339 g/ml, at 100,000 *g* in a Beckman SW50 rotor

for at least 24 h.

b) the frozen pellet was suspended in 30 ml of a mixture of Freon 113 (Dupont) and PBS (one part Freon + two parts PBS) and mixed in a Sorvall Omni-mixer (Dupont Instruments, Wilmington, Delaware) for 1 min, and virus was extracted as described above.

6. Virus Assay

Viral infectivity was measured by two different methods:

a) The 50% tissue culture infectious dose ($TCID_{50}$) assay for equine adenoviruses was carried out in tube cultures of equine primary kidney cells in MEM, supplemented with 2% calf serum. Serial ten-fold dilutions of CsCl purified virus were dialyzed versus PBS at 4° C for 3 h, and diluted in MEM (minus calf serum). Four tube cultures were inoculated with 0.1 ml of each dilution. Virus was adsorbed for 60 min at 37° C, then 2 ml of maintenance medium were added to each tube and cultures were incubated at 37° C on a Labline roller apparatus (Labline Instruments Inc., Melrose Park, Ill.). The cell monolayer was examined daily for CPE for 7 days after infection and the log $TCID_{50}$ was calculated by the Karberg method (1931).

b) Plaque assays for equine adenoviruses were performed using a modification of the technique of Williams (1970). Equine primary kidney cells, 1×10^5 cell/ml, passage 1 to 4, were seeded onto disposable 60 mm diameter tissue culture dishes and maintained in growth medium at 37° C in a 5% CO_2 atmosphere. The cell monolayers were washed with 5 ml of MEM (minus calf serum), and serial ten-fold dilutions of purified virus were prepared, and 0.3 ml of each dilution was

inoculated into duplicate cultures. Virus was adsorbed at 37° C for 60 min and the plates were rotated at 15 min intervals. After the adsorption period the cell monolayers were overlayed with melted agar medium prepared as follows:

A: 1.8% Noble agar (Difco) in distilled water was melted and kept at 43° C;

B: 2x MEM, without phenol red and containing two times concentration of growth medium additives and 1 ml of 50 mM $MgCl_2$, kept at 37° C.

The cell monolayers were overlayed with 5 ml of (1 part A: 1 part B). After overlay, cell plates, including controls, were inverted and incubated at 37°C in an atmosphere of 5% CO_2. Five days PI, 5 ml of similar overlay was added and on the seventh day PI a third overlay containing 1:10,000 neutral red was added. The plates were incubated overnight and examined for plaques.

7. Preparation of Hyperimmune Sera

Purified equine adenovirus virions from equilibrium centrifugation were obtained as described above. The relative amount of viral protein for each isolate was determined by spectrophotometric measurement (1 OD_{260} ≡ 0.283 mg viral protein; Dr. R. Marusyk, personal communication). 0.5 ml of purified virus containing 40 µg protein/ml was dialyzed versus PBS at 4° C for 3 h and mixed with an equal volume of complete Freund's adjuvant and injected intramuscularly into a rabbit. One week after first injection, the second booster, which was prepared as above, was given via the same route and five weeks later an additional injection of 1 ml of purified virion (less adjuvant) was given intravenously. All animals were exanguinated and the sera collected separately for each

equine adenovirus isolate, inactivated at 56° C for 30 min and stored at -35° C before use.

8. Serum Neutralization and Neutralization Enhancement Assays

a) Serum Neutralization

The 50% TCID endpoint of each virus isolate was determined as described above and 10 to 100 $TCID_{50}$/0.1 ml of virus was used in each test as a test dose antigen. Two-fold dilutions of each antiserum were prepared in MEM (less calf serum) and 0.5 ml of test dose antigen was added to an equal volume of each antiserum dilution, mixed well and incubated at room temperature for 60 min. From each antigen-antibody mixture a 0.1 ml aliquot was inoculated into 3 tubes of equine primary kidney cells, the virus adsorbed for 60 min at 37° C and then 2 ml of maintenance media was added to each tube and the tubes incubated at 37° C. Cultures were examined daily for determination of the neutralizing titer for each antiserum.

b) Neutralization Enhancement

Serial two-fold dilutions of each antiserum were prepared as described above, and were mixed with 100 $TCID_{50}$/0.1 ml of test-dose antigen (1:1) and incubated at room temperature for 1 h. To this mixture of antigen-antibody was added 0.1 ml of a dilution of 1:2 to 1:20 of anti-globulin serum (goat anti-rabbit IgG; Cappel Laboratories Cochranville, PA., U.S.A.) and reincubated at room temperature and 4° C for 1 and 18 h respectively. A 0.1 ml aliquot from each mixture was inoculated into two tubes of equine primary kidney cells and treated as described for the serum neutralization assay. Cultures were examined

over a 14 day period for determination of the neutralization enhancement titer.

9. Hemagglutination and Hemagglutination Inhibition Assays

a) Hemagglutination:

Serial two-fold dilutions of purified virions in CsCl were prepared in PBS, using plastic microtiter plates (Cooke Engineering Company, Alexandria, VA., U.S.A.). To each 50 ul of diluted antigen was added an equal volume of a 1% suspension of human group "O" erythrocytes in PBS, and the plates were incubated at 37° C for 1 h. The hemagglutination titer was taken as the highest dilution of virus which produced complete agglutination of the erythrocytes. The results recorded as (+), complete agglutination, p, partial agglutination, or (-), negative agglutination.

b) Hemagglutination inhibition:

Two-fold serial dilutions of each antiserum were prepared in PBS and to each dilution an equal volume of 25 ul of a test-dose antigen (8 HA units) was added, mixed and incubated at 37° C for 1 h. To each dilution, 50 ul of a 1% suspension of human "O" erythrocytes was added and the plates were reincubated for an additional 1 h at 37° C. Plates were examined after each incubation period for determination of the hemagglutination inhibition (HI) titer of each antiserum.

10. Growth Curve of Equine Adenovirus on Equine Primary Cells

Monolayers of equine primary kidney cells in 25 ml plastic

bottles were washed with several volumes of PBS and infected with 2 ml of a 1:1000 dilution of purified virus (MOI~3 $TCID_{50}$/cell). The virus was allowed to adsorb for 60 min at 37° C. The unadsorbed virus was removed by washing the monolayers with 10 ml of prewarmed medium and the cells were incubated in maintenance medium at 37° C. From 0 to 32h PI samples were taken at 4 h intervals and the supernatant separated from the cell monolayer. The cells were scraped off the bottles with a rubber policeman and suspended in 5 ml of MEM (less calf serum), and kept frozen at -35° C before use.

11. Susceptibility of Different Cell Culture Species to Equine Adenovirus

The following cell cultures were grown in 75 ml plastic tissue culture flasks and infected with 3 ml of crude equine adenovirus (MOI~5 $TCID_{50}$):

1 - Equine primary kidney cells (prepared as above)

2 - Feline primary kidney cells (prepared as Equine Primary kidney)

3 - Rabbit primary kidney cells (prepared as above)

4 - Equine dermis cell line (obtained from Dr. J. Hackett, Cell Culture Division of the Naval Biomedical Lab., Oakland, Calif., U.S.A.)

5 - VERO cell line (American Type Culture Collection)(CCL-81)

6 - MDCK cell line ATCC (CCL-34)

7 - Human foreskin cell line (Provincial Laboratory, Division of Virology, University of Alberta)

8 - BHK-21 cell line ATCC (CCL-10)

Virus was adsorbed for 60 min at 37° C and the cells were incubated in maintenance media at 37° C until CPE was observed. Samples from the degenerating host cell systems were titrated by hemagglutination and infectivity assays.

12. UV Absorption Spectrum of Purified Equine Adenovirus

The maximum ultra-violet (UV) absorbance of purified equine adenovirus from CsCl equilibrium centrifugation was determined between the wave lengths 240 to 320 nm. Measurements were carried out on a Pye-Unicam SP1800 recording ultraviolet spectrophotometer (Canlab, Edmonton, Alberta).

13. Sucrose Gradient Centrifugation of Equine Adenovirus Soluble Components

Material collected from the region above the band of complete virions (soluble components) was pooled and concentrated ten times by forced dialysis against polyethylene glycol 6000 (PEG-6000; Baker Chemicals). The concentrated material was then dialyzed against buffer (0.05M Tris-Hcl, pH 8.4) at 4° C overnight, and was layered on top of a preformed gradient of 5-20% sucrose in the same buffer and centrifuged at 70,000 *g* for 30 h using a SW27 rotor. Fractions were collected dropwise from the bottom of the tube in aliquots of 5 µl and tested for the presence of hexon antigen by immunoelectrophoresis against hetero- and homotypic antisera. Zonal centrifugation on sucrose gradients was also used for separation of components carrying soluble

complete hemagglutinins, such as penton and fiber dimers (oligomeric structures) and penton and fiber monomers (incomplete hemagglutinins).

14. Ion-exchange Chromatography

DEAE-Cellulose (Sigma Chemical Company, St. Louis, Mo.) was prepared as a 20% (w/v) suspension. The suspension was washed with 600 ml 0.5 N HCl followed by 600 ml 0.5 N NaOH for 1 h. The washing was continued with several volumes of 0.05 M Tris-H 1, pH 8.4. Chromatography columns of 1.6 x 30 cm were prepared by standard methods. Each chromatographic material was packed by gravity and then washed with 50 ml of equilibrating buffer. Hexon-containing fractions from sucrose gradient centrifugation were pooled, concentrated and dialyzed overnight as described above and layered on the top of the column. Fractions were eluted from the column in a 2 ml volume using a 0 to 0.8 M NaCl gradient in 0.05 Tris-HCl, pH 8.4, buffer. Resistivity of each fraction was measured with a conductivity salt bridge apparatus (Model 31, Yellow Springs Instruments, Yellow Springs, Ohio) and the molarity of each fraction was determined using a computer-generated "best fit" (least squares) straight line program.

15. Detection of Virion Associated Endonuclease Activity

Purified virus was prepared as described above and dialyzed versus distilled water for 60 min, followed by dialysis against 0.005 M Tris-maleate buffer, pH 6.2, for 20 h at 4° C. The dialysate was submitted to centrifugation at 80,000 *g* for 90 min and the supernatant was used as a source of virion-derived pentons. Endonuclease assays

were then performed using the fluorimetric assay procedure described by Marusyk et al. (1975).

16. Agar Gel Immunoelectrophoresis and Micro Gel Double Diffusion

a) Agar gel immunoelectrophoresis plates were prepared by adding 12 ml of melted agarose (BioRad Laboratories, Richmond, CA.), 1% (w/v) in 0.1 M Veronal buffer, pH 8.6, to 8 x 10 cm glass plates. After the agar had solidified sample wells were made by means of a 3 mm diameter gel puncher, plastic template and guide. Two rows of 19 wells were punched side by side, for counter immunoelectrophoresis, with a 5 mm center to center inter-well distance, for maximum interaction between antibody and antigen. After addition of 5 ul of antigen to each lower row well and 5 ul of antibody to the upper row wells, the plates were placed in the electrophoresis apparatus (BioRad Laboratories, Model 1400 Electrophoresis Cell, Richmond, CA.) and electrophoresis was carried out at a constant current of 12 mA for 90 min on a water cooled bed. The buffer-gel connection consisted of "Telfa" paper wicks. At the end of each electrophoretic analysis the glass plates were treated as follows:

1) The gel was covered with a layer of filter paper and a 3 cm of layer of blotting paper, and pressed for 15 min; 2) The filter paper was removed and the gel dried with a hot air blower for 10 min; 3) The plates were placed in staining solution (2% Coomassie brilliant blue R in acetic acid: methanol: water, 7:5:88 respectively) for 10 min; 4) The plates were destained at least two times for a period of 10 min, pressed and dried as described above.

b) Micro gel double diffusion plates were prepared by adding 5 ml of 1% (w/v) melted agarose in PBS to 5 x 5 cm glass slides. After the agar had solidified, wells were punched using a 3 mm gel puncher, with a 2 mm center to center inter-well distance. 5 ul antibody or antigen were added to appropriate wells and the gel was incubated for 24 to 72 h at room temperature in a humidified chamber. After precipitation lines were observed, the gel was pressed and stained as described above.

17. Buoyant Density of Virion

The buoyant density of equine adenoviruses was determined in the following manner. Purified virus from density gradient centrifugation was layered on the top of CsCl (initial density, 1.339 g /ml), and centrifuged to equilibrium for 48 h in a SW50 rotor at 100,000 *g* at 4° C. Fractions were collected from the bottom of each tube. The buoyant density of each fraction was determined by weighing a 100 ul aliquot on an analytical balance. An equal volume of ion-exchange water was also weighed and the buoyant density of each fraction was calculated. The hemagglutination titer and optical density (260 nm) for the fractions collected were also determined.

18. SDS-Polyacryamide Gel Electrophoresis of Virion Proteins

a) Preparation of stacking and resolving gel: a modification of the procedure outlined by Maizel (1971) was used for polypeptide analysis. Using a BioRad Model 220 apparatus, 150 x 100 x 1.5 mm polyacryamide slab gels were prepared, each consisting of a 3% stacking

gel above a 13% resolving gel (acrylamide-bisacrylamide ratio of 30:0.8). The resolving gel was poured first, and after polymerization, a slotted comb was inserted into the apparatus and the stacking gel was added. Air bubbles were carefully removed. After polymerization, the comb was removed, and the slots were overlayed with electrode buffer.

b) Preparation of samples of purified virus: purified virus protein concentrations were determined by spectrophotometric measurement (see above) and for virus components by the BioRad protein assay system (Bradford, 1976). All samples were dialyzed against distilled water at 4° C for 2 h to remove salts, and then lyophilized under vacuum at -70 ° C overnight. 50-100 ug lyophilized viral protein samples were resuspended in 100 ul dissociating buffer containing a final concentration of 0.05 M Tris-Hcl, pH 7.6, 1% sodium dodecyl sulfate, 0.1% β-Mercaptoethanol, 10% glycerol and 0.002% bromophenol blue, and incubated at room temperature for 2 h. The samples were then subjected to electrophoresis for 240 min at a constant current of 30 mA. The slab gels were removed and stained overnight in 0.2% Coomassie brilliant blue R in methanol-acetic acid-water as described above and destained in the same solution (less stain) until clear. With reference to the relative mobility of HAd-2 virion polypeptides of known molecular weight (Anderson et al, 1973; Weber, 1976) in the same gel, the molecular weights of the resolved equine adenoviruses polypeptides were calculated by using a computer-generated "best fit" straight line program.

c) Densitometer tracings of Coomassie blue stained gels: the slices were obtained by slicing the slab gels lengthwise into

appropriate size, with each strip containing the resolved polypeptide bands of each sample. The resolved polypeptides were scanned at 620 nm in a Gilford 240 spectrophotometer with a linear transport scanner.

19. Stability of Virion in CsCl

The stability of equine adenovirus in CsCl at 4°C and room temperature was determined. Aliquots of purified virus were kept in CsCl, p = 1.339 g/ml (3.06M, in 0.05M Tris-HCl, pH 8.0). From 0 to 6 weeks post purification samples were taken at one week intervals and tested for hemagglutination activity as described above.

20. pH Stability of Virion

The stability of equine adenovirus in buffers ranging from pH 2 to 11 was determined as follows: 2 ml of purified virus in CsCl (HA titer = 128) was suspended in 19 ml of PBS and then 2 ml aliquots were dialyzed for 1 h at room temperature against the following buffer solutions: pH 2.0 to 8.0 (0.2 M Na_2HPO_4 and 0.1 M citric acid); pH 9, 10, 10.5 and 11 (0.05 M glycine-NaOH). All samples were then dialyzed against neutralizing buffer, pH 7.0, overnight. The hemagglutination titer of each sample was determined as described above.

21. Thermal Denaturation of Purified Viral DNA

Equine adenovirus nucleic acid extraction was carried out using the method of Doerfler et al (1972). Purified virus in CsCl was dialyzed against 0.02 M Tris-HCl, pH 7.5, for 1 h at 4° C, and then

incubated with 500 ug/ml of pronase, 0.4% SDS, and 0.002 M EDTA at 37° C for 1 h. The DNA was extracted three times with either two volumes of redistilled phenol, saturated in 1 M Tris-HCl, pH 7.5, or chloroform:butanol (3:1). The aqueous layer was separated by low speed centrifugation and dialyzed against SSC buffer (0.15 M sodium chloride + 0.015 M sodium citrate) overnight at 4° C. All measurements were carried out with a Pye-Unicam SP1800 UV recording spectrophotometer attached to Unicam AR-25 linear recorder.

22. Electron Microscopy Studies

a) Negative Staining

Electron microscopic examination of equine adenovirus preparations was performed on a Philips EM300 or Siemens Elmiskop-102 electron microscope operating with a beam acceleration of 80 KV. Purified virus in CsCl was drop-dialyzed versus 0.1 M ammonium acetate and a drop of dialyzed virus was placed on a carbon-coated 400 mesh copper grid. The excess liquid was removed with a filter paper and a drop of 3% sodium silicotungstic acid (SSTA) immediately added to each grid. After 15 seconds the excess stain was removed as above and the grids dried and examined in the microscope. The length of the virion fiber component was calculated by comparison with the diameter of observed virions on enlarged photographic images.

b) Electron Microscopy of Purified Viral DNA

Purified equine adenovirus in CsCl was disrupted by addition of an equal volume of 6 M guanidine hydrochloride and incubated at 37° C for 1 h (the final concentration of guanidine hydrochloride was 3 M).

This treatment disrupted the virus and gently released viral DNA. For electron microscopic examination the aqueous technique of Kleinschmidt (1968) was used. The spreading solution consisted of 100 ug/ml cytochrome C in 0.5 M ammonium acetate, approximately 2 ug/ml of DNA and 0.01 M EDTA. The hypophase was 0.25 M ammonium acetate and 0.01 M EDTA. The grids with DNA were stained with uranyl acetate. The contour length of DNA preparations was determined on enlarged photographs with a map tracer. The molecular weight of DNA was calculated using the technique of Lang (1970). For shadowing, grids containing DNA were shadowed with platinum-palladium at a 15 degree angle in a vacuum evaporator apparatus (NRC-3115, Varian Vacuum Division, Calgary, Alta.) and examined in the electron microscope.

c) Thin Sectioning of Equine Adenovirus

After infection of monolayers of equine primary kidney cells in plastic flasks at a multiplicity of approximately 5 $TCID_{50}$/cell, samples were taken at 48 h PI. The medium was discarded and cell monolayers were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 at 4° C for 2 h. The cells were gently scraped off the culture vessel surface with a rubber policeman, washed, and postfixed in osmium tetroxide, and embedded in Epon 812. Thin sections were cut with a diamond knife and mounted on acid-cleaned copper grids. The grids were stained with 5% uranyl acetate in methanol followed by staining in lead citrate (Reynolds, 1963) and examined by electron microscopy.

23. Buffer Solutions

Buffer solutions required for the course of this study were prepared from tables listed by Gomori (1955).

RESULTS

1. Cytopathology and morphological alterations produced by an EAdV in cultured fetal equine primary cells as observed by light and electron microscopy.

Initial CPE was observed after 16 h PI. Foci of cytopathology consisted of groups of rounded cells which were highly refractive. The characteristic epithelial morphology (Fig. 1) degenerated as cells became rounded. The progression of CPE to detachment of the cells occurred between 48 to 72 h PI (Fig. 2). In monolayers of EPK cells, initial changes were observed 20 h after infection. Hematoxylin-eosin staining of infected cells at 48 h PI showed a characteristic rounding of cells with either single or multiple basophilic inclusion bodies and a shrinkage of chromatin. No changes were observed within the cytoplasm of infected cells. All control cell monolayers showed active cell division after 48 h. Electron micrographs of infected cells in thin section at 48 h PI revealed viral particles in a lattice arrangement with margination of the nuclear chromatin and disintegration of the nuclear membrane in some areas. A few viral particles were seen in the cytoplasm (Fig. 3). The assembly and arrangement of virus particles in the nuclei were also of special interest at 48 h PI. In some electron micrographs (Fig. 4) the center to center distance between virus particles in parallel rows of the lattice was found to be greater than expected (this distance is usually equal to the diameter of one virus particle, i.e. 75-80 nm). Some virus-induced intranuclear inclusion bodies were also present 48 h PI. No intranuclear protein crystals were observed in any of the electron micrographs taken of the four equine adenovirus

Figure 1

EPK cells stained with hematoxylin-eosin

Equine primary kidney cells were grown in Leighton tubes at 37°C. The cells were fixed with Zenker's solution for 24 h at room temperature and stained with hematoxylin-eosin. Epithelial morphology is evident. (X 428)

Figure 2

EPK cells infected with EAdV and stained with hematoxylin-eosin

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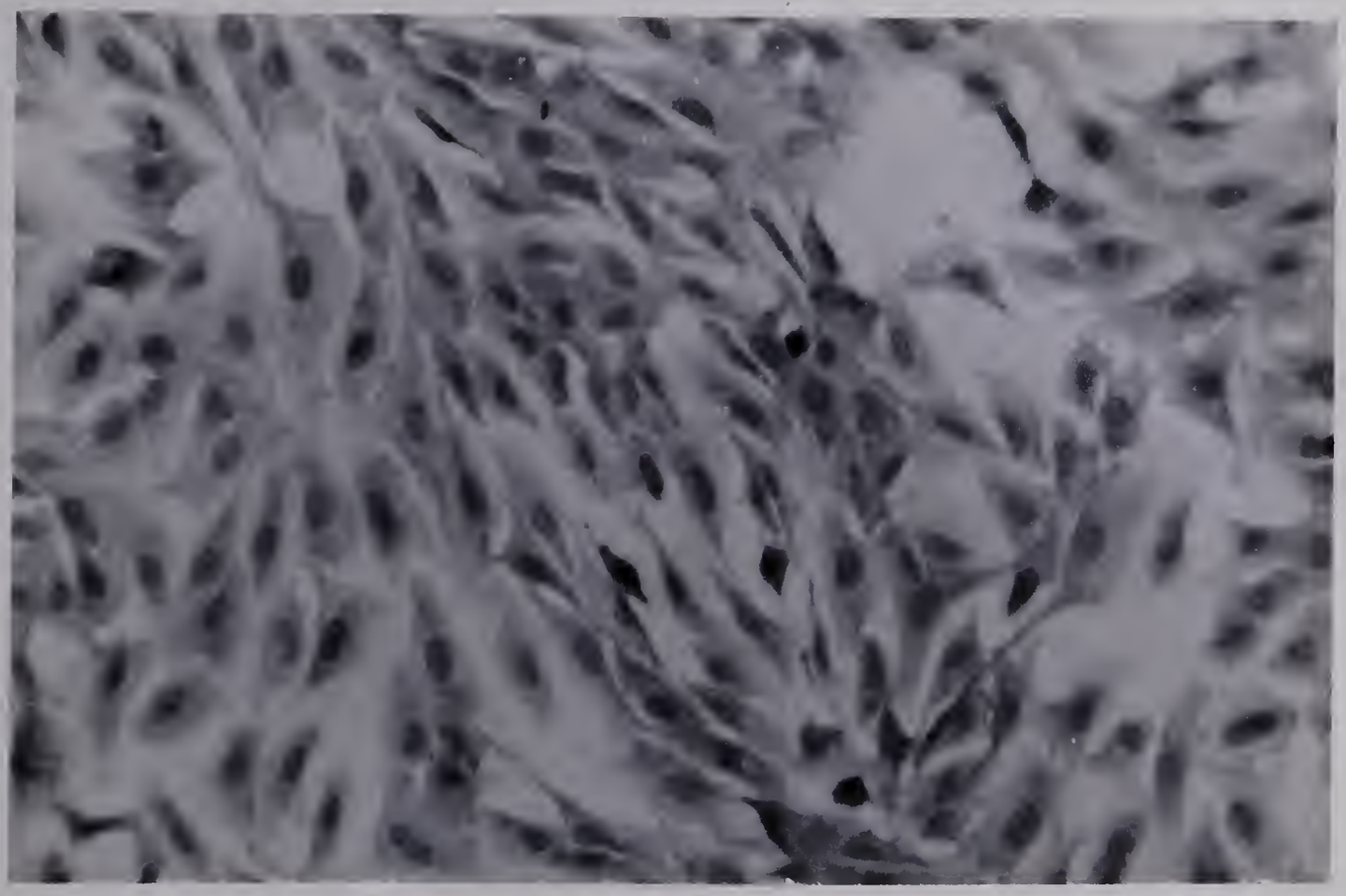
Confluent cultures of EPK cells in Leighton tubes were inoculated with 0.3 ml of EAdV (MOI~5 to 10 $TCID_{50}$/cell). Cells were fixed with Zenker's solution 48 h PI and stained with hematoxylin-eosin. Notice the discrete rounding of cells. (X 428)



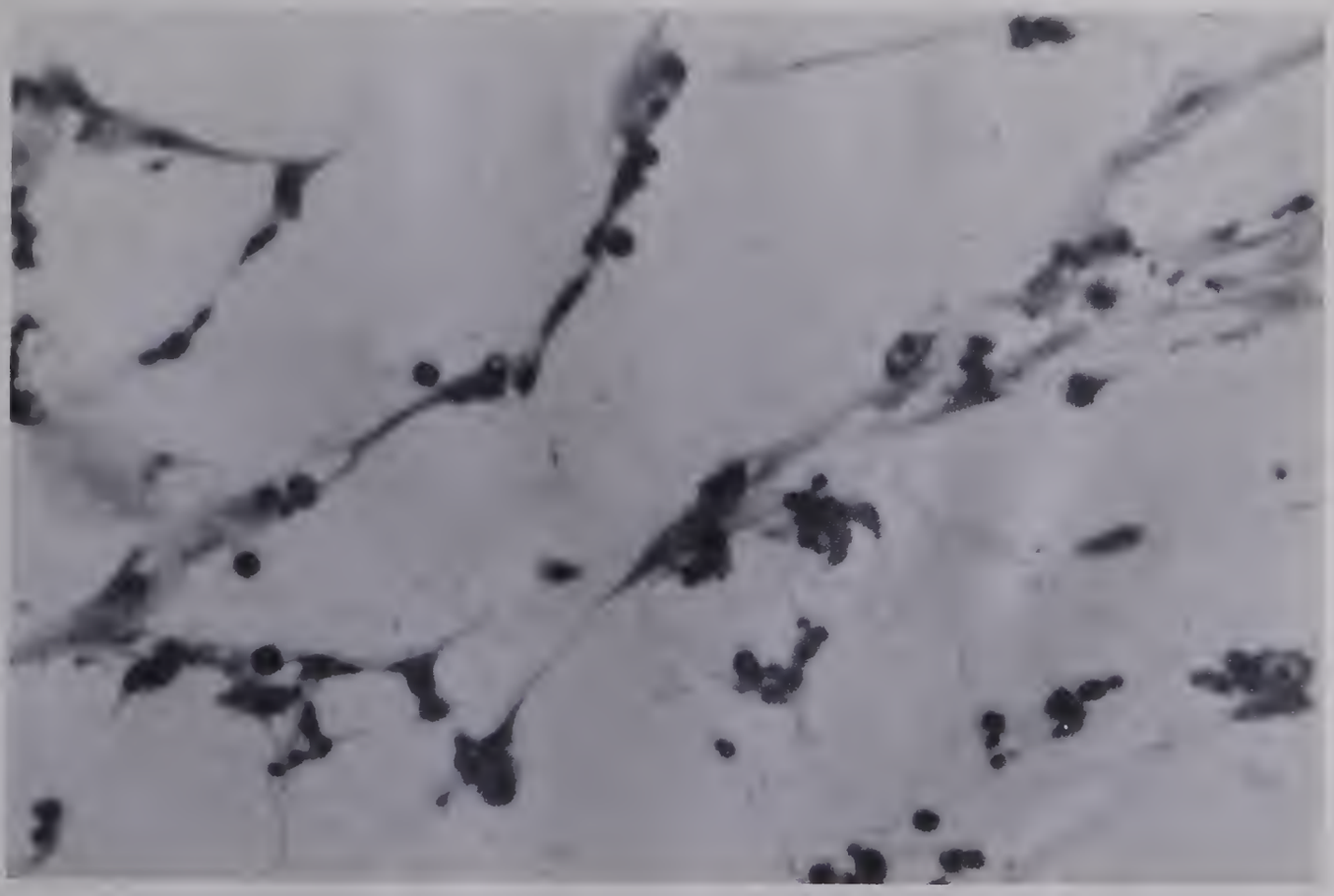


Figure 3

Electron Photomicrograph section of EPK cells infected with EAdV

EPK cells were infected with EAdV (MOI~5-10 $TCID_{50}$/cell). Forty-eight h PI cells from infected cultures were scraped off with rubber policeman and pelleted by centrifugation. The cell pellets were fixed with 3% glutaraldehyde, postfixed with osmium tetroxide, dehydrated and embedded as described in Materials and Methods. The thin sections were stained with uranyl acetate and lead citrate. The crystalline lattice arrangement of viral particles and disintegration of the nuclear membrane are seen.

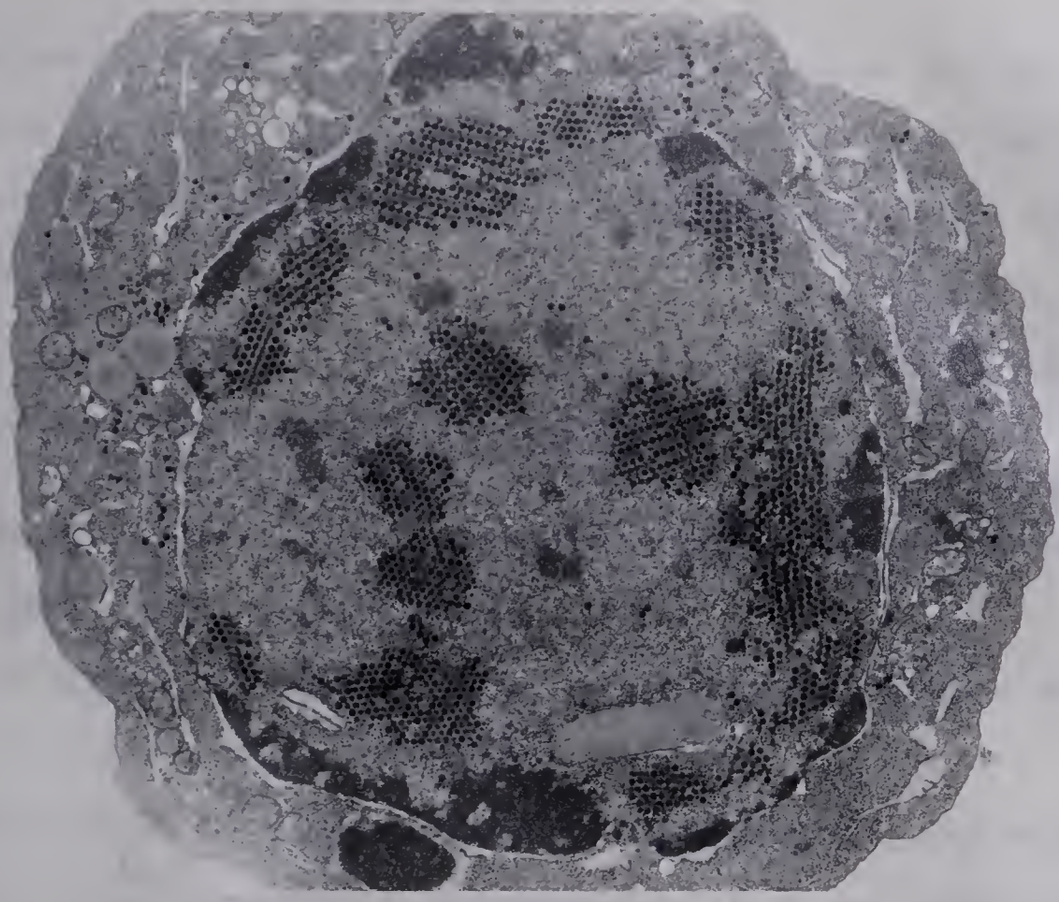
Magnification: X 21,711

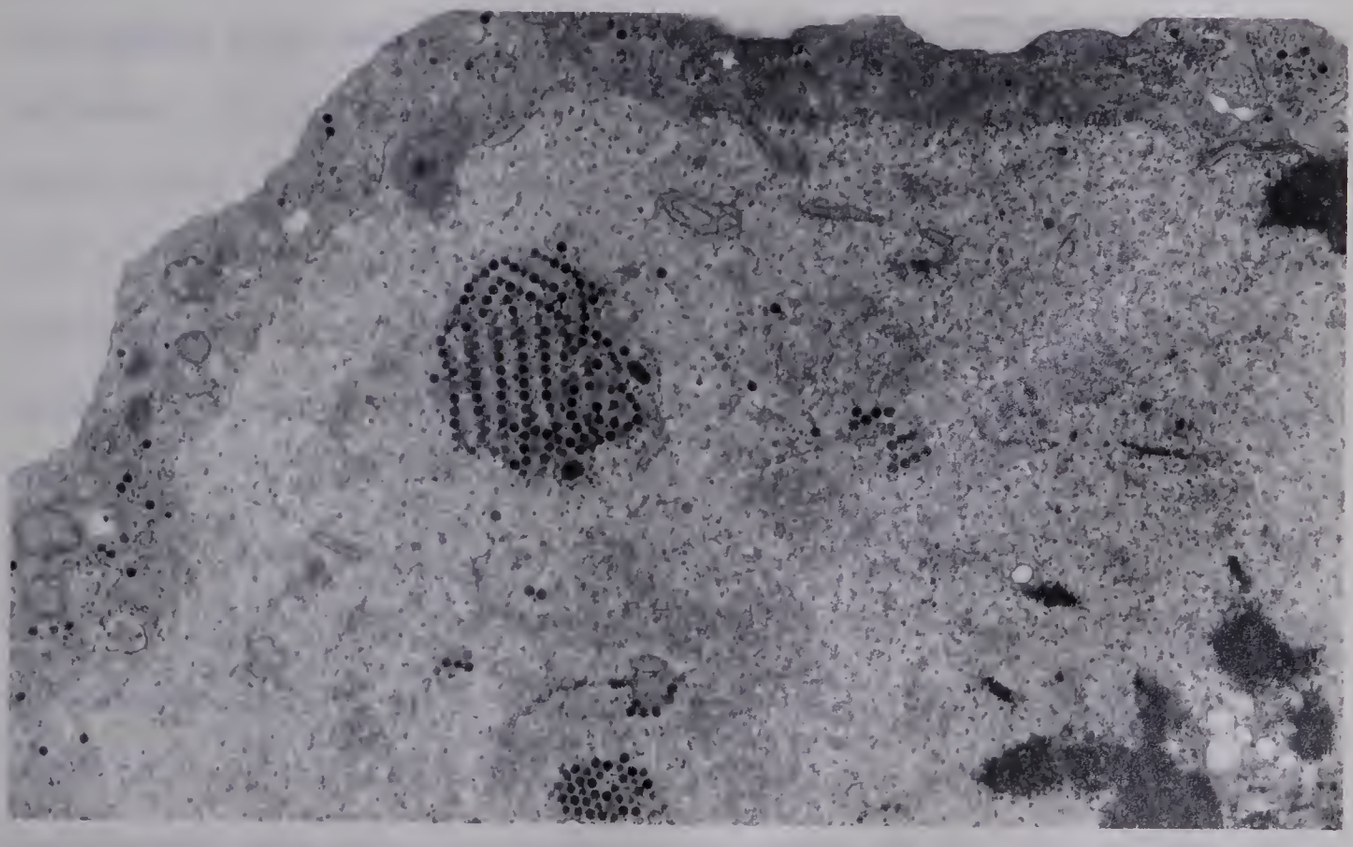
Figure 4

Electron Photomicrograph section of EPK cells infected with EAdV

The experiment was performed as described in Fig. 3. Note the unusual linear arrangement of the viral particles.

Magnification: X 27,900





isolates, as has been observed with human adenoviruses at the same interval PI (Carstens, 1974). All viral particles within the nuclei of infected cells were hexagonal in outline indicating icosahedral symmetry of the virion.

2. Purification and Concentration of EAdV

Several methods can be employed for purification and concentration of the equine adenoviruses.

i) Freeze-thawing of infected cells three to five times at -35°C and room temperature.

ii) Sodium deoxycholate treatment of degenerated cells at pH 9.0.

iii) Freon extraction of degenerated cells at room temperature.

Each of the above methods is followed by two step CsCl centrifugation. For optimal virus recovery, a combination of the above methods was employed. It was found that the combination of freezing and thawing for three cycles at room temperature and -35°C, followed by 1 h extraction with 0.2% sodium deoxycholate at the same temperature and two cycles of centrifugation in a CsCl, led to maximal recovery and concentration of virus as observed by electron microscopy, infectivity and hemagglutination tests. An attempt was also made to purify the virus by Freon extraction, using a mechanical homogenizer, followed by removal of cellular debris and CsCl centrifugation as above. When virus infectivity and hemagglutination tests were performed, it was found that more infectious particles could be recovered by gentle methods, e.g. sodium deoxycholate extraction, in comparison with the Freon method. The virus band, after isopycnic centrifugation in CsCl, was clearly evident,

without cellular debris. Electron microscopic examination of an aliquot from the purified virus preparation showed intact adenovirus particles (Fig. 5 and 6).

3. Plaque Production in EPK Cells

Passage three to four of equine primary kidney cells were found to be the most susceptible passage level for production of plaques with all equine adenovirus isolates. Addition of 10% (w/v) lactalbumin hydrolysate to the overlay media was found to be essential for maintaining the cells in a viable state necessary for the production of plaques. It was also found that the addition of magnesium chloride (Williams, 1970) at 50 mM concentration in the overlay medium was crucial for enhancement of plaque formation. Plaque formation usually occurred 7 to 8 days PI for all EAdV isolates. The average diameter of individual plaques was 3.3 mm. The plaque size usually increased 24 h after the last overlay (containing neutral red). The plaque morphology and plaque size among different equine adenoviruses were found to be similar when observed at a standard time after the last staining overlay (Fig. 7). Every individual plaque had a circular clear zone with a partially smooth edge. No plaques were produced when crude virus (virus from 3 cycles of freeze-thawing and centrifugation at 800 g for 15 min) was used. A titer of 6×10^6 PFU/ml ($\sim 10^{6.5}$ $TCID_{50}$/ml) was found to be equivalent to approximately 200 ug protein in 1 ml of purified virus suspension by spectrophotometric measurement.

Figure 5

CsCl isopycnic centrifugation of sodium deoxycholate extracted EAdV

Virus was purified and concentrated by CsCl isopycnic centrifugation (70,000 g) for 24 h in a SW50 rotor. The virus band in each tube is evident.

Figure 6

Electron photomicrograph of EAdV negatively stained with sodium silicotungstate

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Virus was purified as in Fig. 5, and stained with 3% sodium silicotungstate, pH 6.8.

Magnification: X 91,330

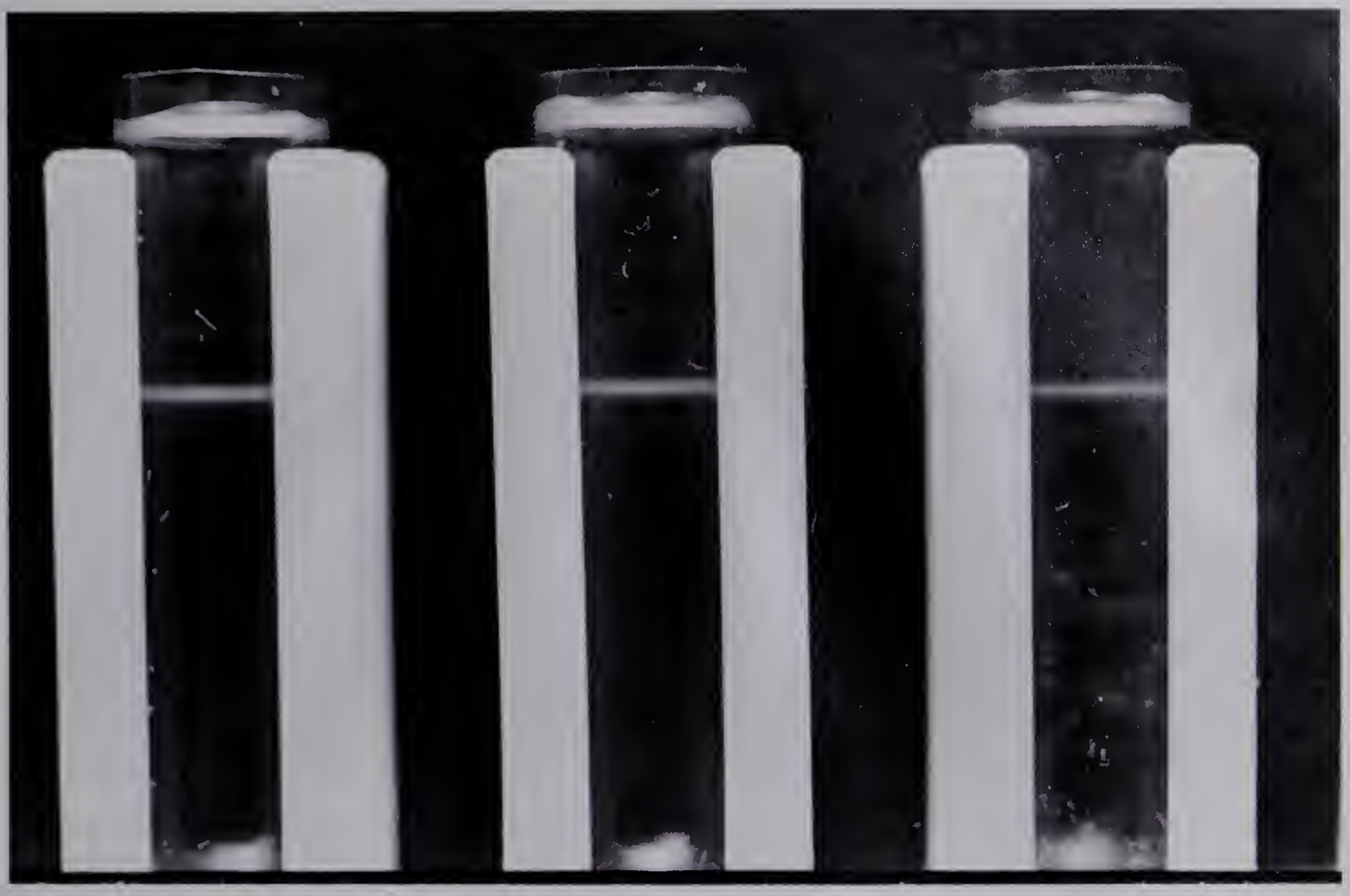


Figure 7

Plaques formed by four EAdV isolates in EPK Cells

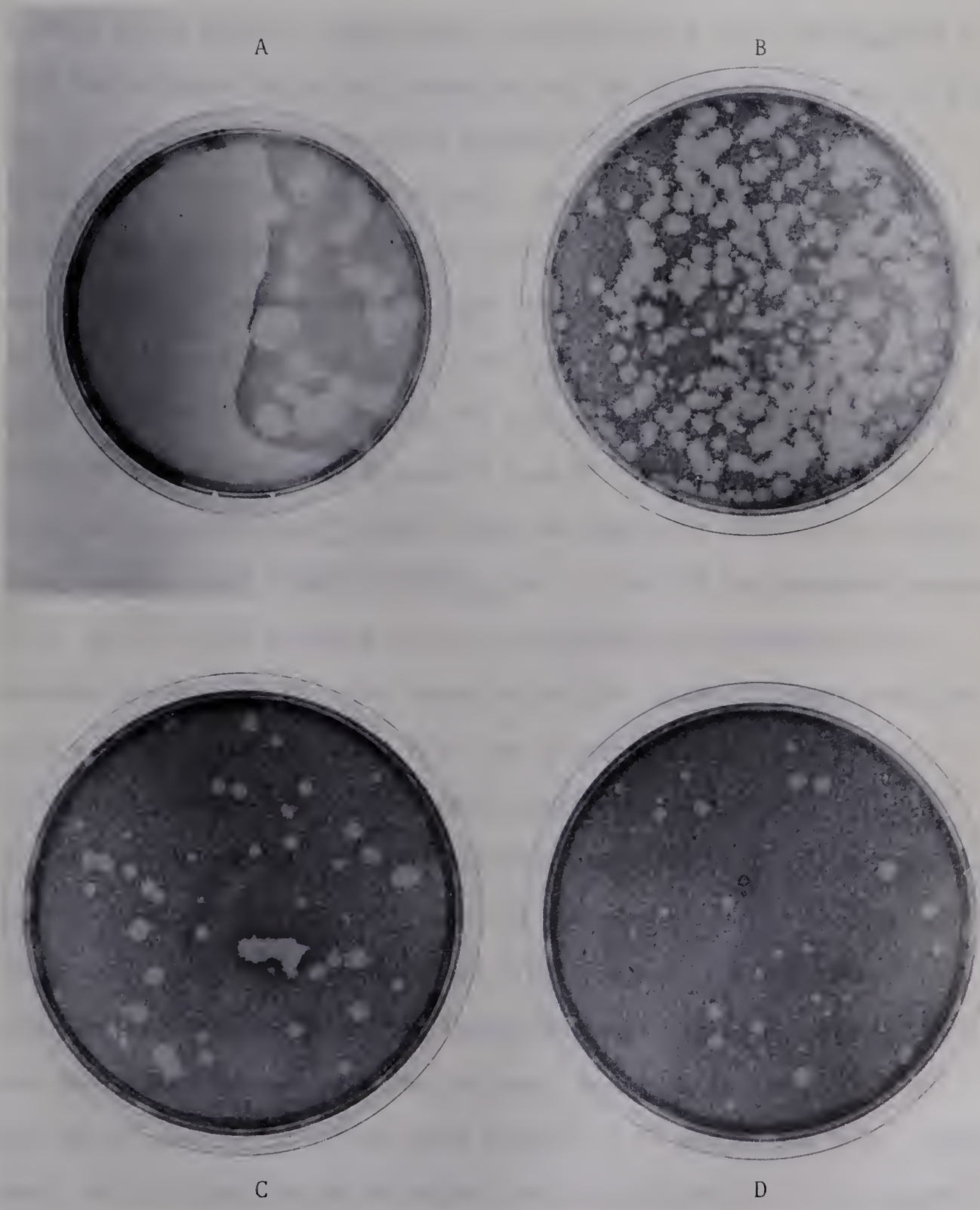
Plaque assay of purified virus from isopycnic centrifugation was performed as described in Materials and Methods.

A - EAdV004, 48 h after last overlay

B - EAdV020, 24 h after last overlay

C - EAdV008, 12 h after last overlay

D - EAdV-Briarwood 12 h after last overlay


A
B
C
D

4. Susceptibility of different cell culture species to EAdV

Monolayers of eight different cell culture systems, including primary cells and cell lines, were infected with 5 to 10 $TCID_{50}$/cell of EAdV and observed daily for production of CPE (Table II). The CPE in passage one of EPK and FPK cell cultures appeared 16 h PI, with rounding of cells and the CPE progressed rapidly. Between 24 to 32 h PI, highly refractile cells began to form in cluster-like structures and the result was a typical "grape-type" CPE, with 90% cell sheet detachment occurring 48 h PI. The type of CPE seen in RPK and the progression of such was much slower than in the EPK and FPK cell cultures at the same passage. Complete cell destruction occured 4 days PI. The MDCK line was also found to be susceptible to EAdV, when sub-confluent (50%) cell culture were infected with a MOI~5-10 $TCID_{50}$/cell. The CPE was apparent between 16 to 20 h PI with rounding of cells, progressing to complete cell destruction at 48 h PI. The formation of CPE in EDC line was achieved with a 1:1000 dilution of 10 $TCID_{50}$/ml purified virus. The fibroblastic morphology of the cells was completely destroyed as cells became rounded and pycnotic degeneration after 48 h PI. The progression of CPE was slower than in EPK when the same concentration of purified virus was used. The production and progression of CPE in Vero cells was usually slow. The pH of the medium rapidly dropped to acidic levels and the cells detached from the surface, usually after 48 h PI. The CPE which appeared in BHK-21 cells after 24 h PI progressed very slowly until 48 h PI, and the pH of medium became acidic 48 h PI. An attempt failed to produce CPE in the HFS cell line and neither hemagglutinin or infectious particles were detected in this system. The highest titer

Table II. The production of cytopathic effect by equine adenovirus isolates in cell culture

Primary cell culture	CPE	Cell lines	CPE
Equine primary kidney	++++	Vero	+
Feline primary kidney	++++	EDC	++
Rabbit primary kidney	++	BKH-21	+
		MDCK	++
		HSK	-

Symbols:
+ Infected cells showed a slight granular change.
++ Infected cells started to round up, with no cell detachment.
+++ 75% of cells became rounded up, with cell detachment.
++++ CPE was observed in 90% of cells, with highly refractile appearance which was followed by rapid degeneration of cell sheet.

of infectious particles was observed with EPK and FPK cells as detected by HA and $TCID_{50}$ tests. EDC and MDCK cell lines were the most susceptible of the cell lines tested. The results of this experiment were in agreement to those reported by Harden (1974).

5. Growth Curve of EAdV

The growth curve of EAdV is shown in Figure 8. A latent period of 12 h was revealed by $TCID_{50}$ assay. This phase was followed by a continuous increase of intracellular and extracellular virus development up to 28 and 32 PI respectively. Hemagglutination activity was also detectable for intracellular and extracellular virus after 16 and 24 PI (the difference in detectability of virus by the two tests is due to the sensitivity of the two assay systems). The shape of the growth curve, when the HA assay was employed for detection of virus, was very similar to a single step growth cycle. The CPE appeared at 16 h PI with rounded cell foci. Complete cell detachment was observed by 40 h PI. The results were in good agreement with those of Harden (1974), who reported a decrease of titer for cell bound virus at 30 h PI and an increase in titer for extracellular virus after 32 h PI.

6. Cross-serum neutralization and neutralization-enhancement of EAdV isolates

Serum neutralization and neutralization-enhancement tests were performed with purified virion and virion specific antisera as described in Materials and Methods. Infected tissue culture tubes were examined daily for production of CPE. The CPE usually appeared between 3 to 4

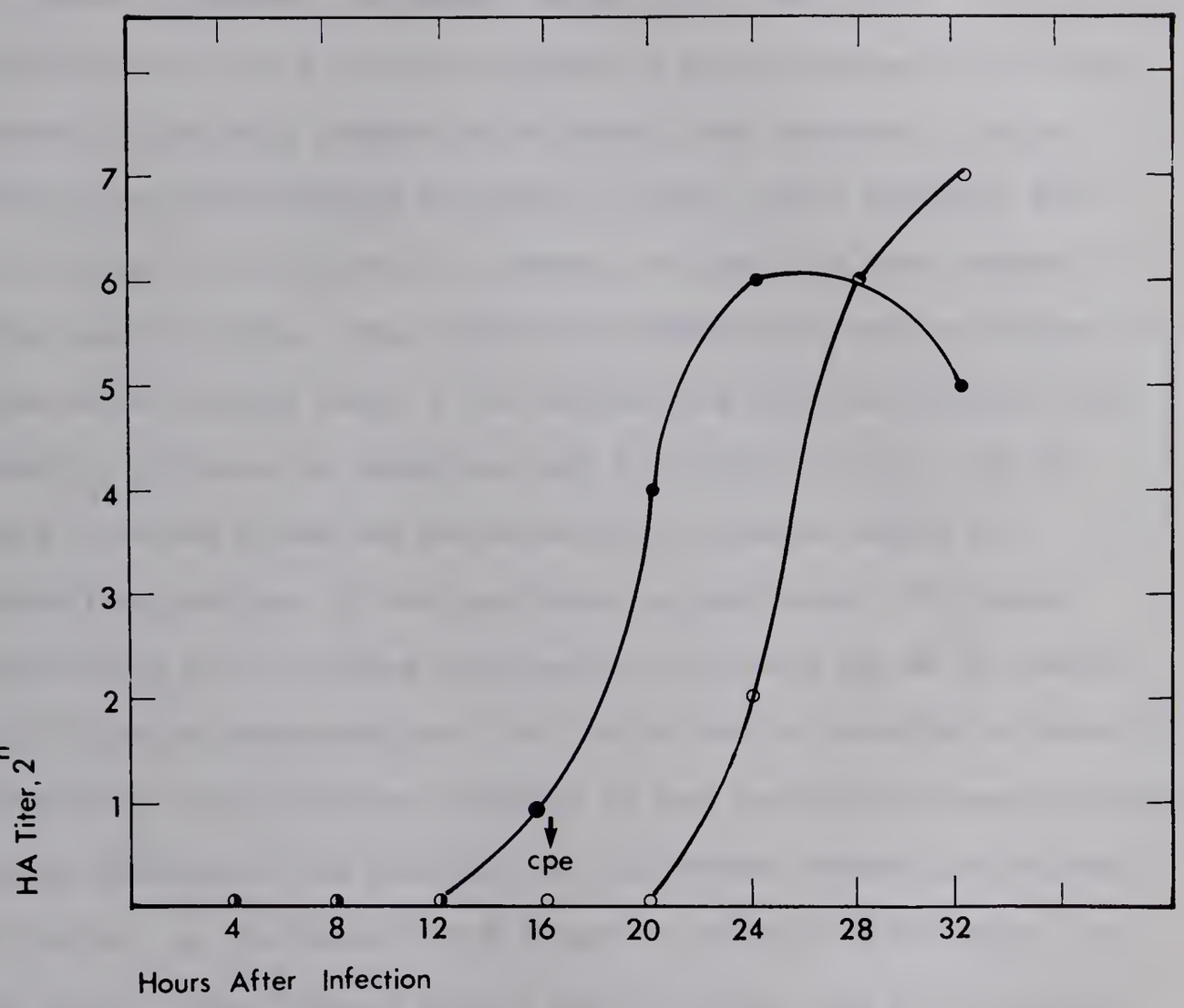
Figure 8

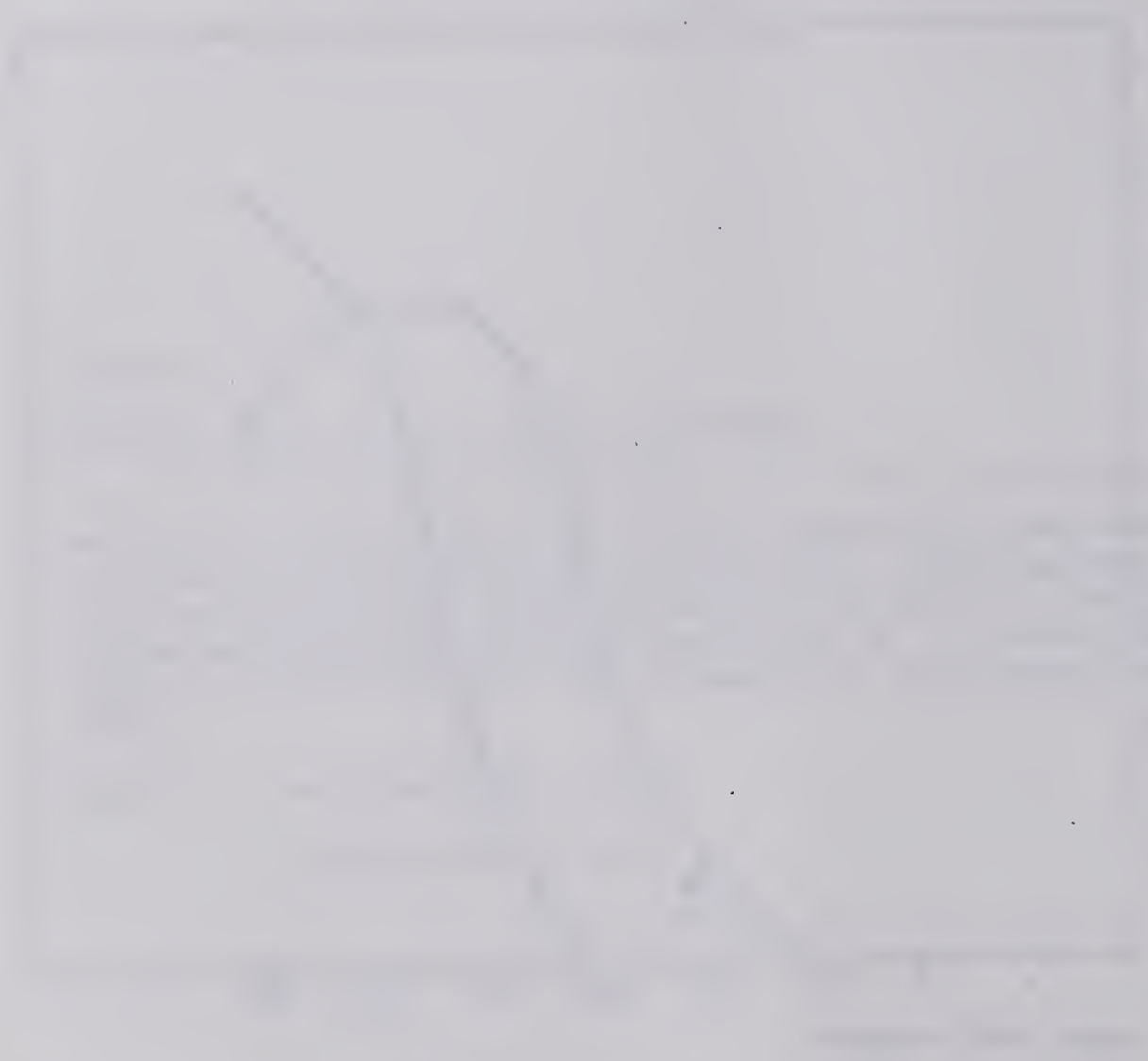
One Step Growth Curve of EAdV in EPK Cells

Monolayers of EPK cells in 3 oz plastic flasks were infected with 1 ml of virus suspension containing 3 $TCID_{50}$/cell. After adsorption, cells were washed with several volumes of PBS and 5 ml of maintenance medium was added and the flasks incubated at 37°C. Samples were taken at selected intervals for intracellular and extracellular virus assay.

Symbols: ● Intracellular virus

○ Extracellular virus


HA Titer, 2^n
7
6
5
4
3
2
1
cpe
4
8
12
16
20
24
28
32
Hours After Infection



days PI, with gradual rounding of cells and progressed at a moderate rate. The neutralization titer for all four antisera was determined on day 7. Further incubation of tubes after the initial 7 day period was not shown to increase the neutralization titer (Table III). A difference of 2- to 4-fold was observed in neutralization titer cross-neutralization tests between the different EAdV isolates. Similar results were also reported by Studd rt (1974), when 6 different EAdV isolated in the United States, Germany, and Australia were compared by serum neutralization. When EAdV020 and EAdV004 were used as antigen in cross-neutralization tests, a low neutralizing titer was obtained with homotypic antisera, in comparison with heterotypic titer. This was found to be due to the low concentration of relative amount of neutralizing antibody in the particular antisera used. The highest neutralizing titer obtained with homotypic antisera was 80 for EAdV008. Neutralization-enhancement was also carried out as described in Materials and Methods, with different dilutions of goat anti-rabbit gamma globulin, taking advantage of the fact that the interaction between sensitizing antibodies, eg. antibodies which attach to antigenic determinants on the virion without causing evident neutralization, can be revealed in the same assay by addition of secondary antibodies (Hahon,1970: Maver et al., 1970; Kjellen & Pereira, 1968; Wadell, 1970; Marusyk, 1972). For a better understanding of the nature of the four-fold difference in cross NT titer obtained between EAdV020 and EAdV008, neutralization-enhancement was carried out, using different dilutions of enhancing sera (1/4 to 1/20). The neutralization-enhancement titer was calculated 14 days PI. The results with this test revealed that after absorption of the hexon-specific antibodies with purified virion, no sensitizing antibodies

Table III. Immunological relationship of equine adenovirus isolates by serum neutralization test

Antigen used	NT titer of antisera against			
	EAdV004	EAdV020	EAdVB	EAdV008
EAdV004	40*	20	80	40
EAdV020	40	40	80	80
EAdVB	40	20	40	40
EAdV008	80	20	80	80

* Reciprocal of highest dilution of antisera that neutralized 10 $TCID_{50}$/0.1 ml of inoculum.

were detectable by neutralization-enhancement. The extent of enhancement of neutralization titer must arbitrarily be at least eight-fold (up to 50% of the non-absorbed titer) to be considered significant (Marusyk, 1972).

7. Hemagglutination, hemagglutination inhibition and hemagglutination inhibition-enhancement

Hemagglutination tests were performed with purified virion and soluble components of the virus for determination of the nature of virion-associated and non-virion-associated hemagglutinin (i.e. complete HA with penton and fiber dimers, incomplete HA with penton and fiber monomers). The hemagglutination titer of purified virions was usually greater than 1024 (HA U/50 ul) with all EAdV isolates.

The hemagglutination activity associated with virions was completely destroyed when purified virus was suspended in PBS and sonicated more than 60 seconds (micro tip, prob, setting 60, Biosonik-III, Bronwill Scientific,N.Y., U.S.A.).When the pooled soluble components of EAdV isolates were highly concentrated against PEG-6000 (500-fold), as described in Materials and Methods, and tested for the presence of oligomeric hemagglutinating structures (penton and fiber dimers), a maximum titer of 32 was obtained. The results showed a very low concentration of these oligomeric structures in the excess pool of virus soluble components. The same results were also obtained when rate-zonal centrifugation and DEAE-cellulose chromatography with concentrated material were carried out (see below). An attempt was also made to detect the presence of monomeric hemagglutinating

structures (penton and fiber monomers) in soluble component, of EAdV isolates by hemagglutination-enhancement tests (Norrby & Skaaret, 1967) Wadell, 1970; Marusyk et al., 1972). Antisera against disrupted HAd6 was used as an enhancing serum (heterotypic antibodies) to reveal hemagglutination by these monovalent structures (which would also reveal an inter-species specificity on the penton base and fiber; Marusyk, 1972). No hemagglutination-enhancement was detected with any of the EAdV isolates. Hemagglutination-inhibition tests were carried out with purified virions. The preliminary results of this test revealed a highly significant difference in HI titer between EAdV020 and three other isolates (Table IV). For further clarification of the high difference in HI titer between isolates, the test was performed with different preparations of purified virions for each isolate and with different lots of hyperimmune sera. Similar results were again obtained. When antisera against EAdV004, EAdV008 and EAdVB were diluted to the level of the highest dilution of antisera against EAdV020 which inhibited hemagglutination with homotypic antigen (in order to insure the same relative amount of HI antibody in each antisera used), the results of cross-HI showed no significant difference in HI capacity among the four EAdV isolates. Hemagglutination-inhibition tests in the presence of different dilutions of goat anti-rabbit antiserum (1/10 to 1/80) were also carried out. No enhancement of HI titer was obtained for EAdV004, EAdV008, EAdVB and EAdV020.

Table IV. Immunological relationship of equine adenovirus isolates by hemagglutination-inhibition test

Antigen used	HI titer of antisera against			
	EAdV004	EAdV020	EAdVB	EAdV008
EAdV004	2560*	80	5120	5120
EAdV020	5120	640	10240	10240
EAdVB	10240	160	10240	10240
EAdV008	20480	160	20480	20480

* Reciprocal of highest dilution of antisera that inhibited hemagglutination by 4 hemagglutination units.

8. UV absorption spectrum of purified EAdV

The adsorption spectrum of EAdV from isopycnic centrifugation in CsCl was determined (Fig. 9). The shape of the curve was very similar to that obtained for a canine adenovirus (Marusyk, 1967). The A260/280 nm ratio was 1.25.

9. Sucrose gradient centrifugation of EAdV soluble components

Rate zonal centrifugation of soluble components of four EAdV isolates was performed for separating the different viral components as described above. EAdV soluble components were concentrated (30- to 50-fold) against PEG-6000 and centrifuged in a preformed linear gradient of 5-20% sucrose. Two different biological activities were identified:

a) complete HA (penton or fiber dimers), in the lower region of the gradient, with a moderate HA titer (16 HAU/50 ul).

b) hexon components were also detected by using counter-immunoelectrophoresis with heterotypic (against HAd3) and homotypic antisera.

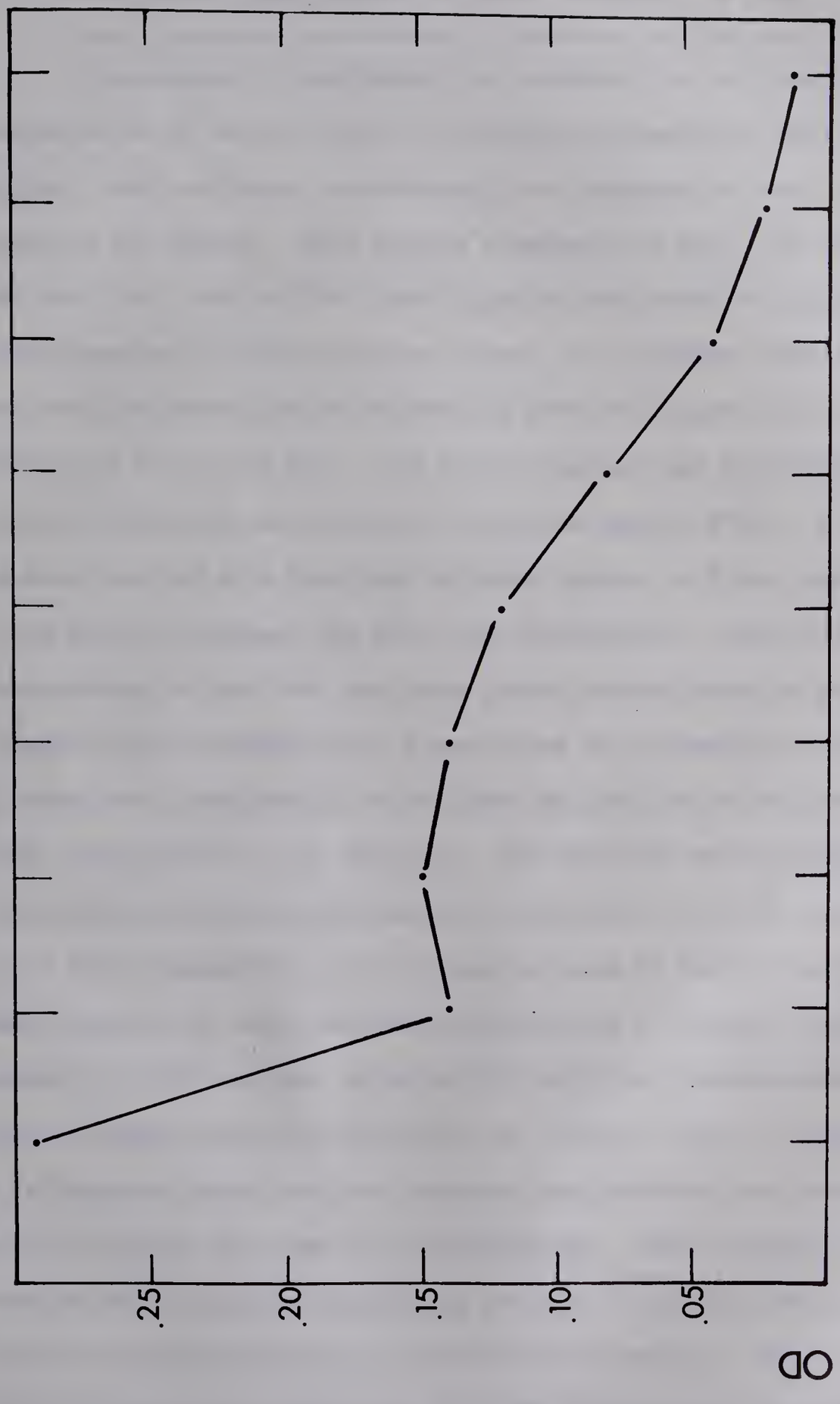
When centrifugation was performed for 30 h at 70,000 g, the hexon usually appeared in the middle of the gradient as a distinct peak. The hexon was also revealed by simple double-gel diffusion using antibody against purified HAd2 hexon and HAd3 virion. The results from the above experiment showed the presence of very low concentrations of oligomeric structures in the soluble component of EAdV, as revealed previously by HA assay. The presence of a low concentration of oligomeric structures in the soluble component population of other adenovirus was reported earlier by Norrby et al. (1971) with simian adenovirus (SA-7).

Figure 9

UV absorption spectrum of EAdV

Purified EAdV was obtained from CsCl isopycnic centrifugation as described in Materials and Methods. The absorbance was measured with a Pye Unicam SP1800 spectrophotometer. The $A_{260/280}$ nm ratio of purified virus was 1.25.

Symbol: ● optical density


.25
.20
.15
.10
.05
OD
240
250
260
270
280
290
300
310
320
Wavelength, nm

10. Ion-exchange Chromatography of hexon components of EAdV isolates

Ion-exchange chromatography was employed for purification and determination of the net charge on the hexon component of each EAdV isolate. DEAE-cellulose chromatography was performed as described in Materials and Methods. When soluble components of each EAdV isolate from the first cycle of CsCl centrifugation were concentrated and chromatographed in DEAE-cellulose columns, two different broad peaks were obtained after elution of material from the column with a linear gradient of 0 to 0.8 M NaCl. The hexon component was associated only with the first peak, as detected by antisera against H^{Ad}-3. A reaction was also detected with homotypic antisera (penton or fiber components) in the fractions between the first and second peaks. When fractions corresponding to the first peak were pooled and subjected to polyacryamide gel electrophoresis, a population of polypeptides was observed, including one corresponding to the hexon polypeptide of reference virus (EAdV) polypeptides in the same gel. The first and second peak from DEAE-cellulose chromatography usually eluted at 0.15 $\pm$ 0.05 and 0.4 $\pm$ 0.05 M NaCl, respectively. An attempt was made to further purify the hexon component by rate zonal centrifugation in a preformed linear gradient of 5-20% sucrose, prior to DEAE-cellulose chromatography, in order to remove protein contamination of cellular origin. After 30 h centrifugation, hexon positive fractions were detected with heterotypic antisera, pooled, and used for chromatography. Only a single peak corresponding to hexon component (as revealed by optical density and counter-immunoelectrophoresis) eluted from the column. The elution profile of hexon was similar for all EAdV isolates, and coincided with

0.18 ± 0.03M NaCl (Fig. 10, 11, 12, 13). The identity of hexon in the fractions from DEAE-cellulose columns was confirmed in polyacryamide gel electrophoresis (PAGE) by the appearance of a single polypeptide at a position relative to the purified EAdV hexon polypeptide.

11. Agar gel immuno-electrophoresis and microgel double diffusion of purified hexon

Group-specific antigenic determinants were revealed in hexons of the four EAdV isolates by using hetero- and homotypic antisera against purified hexon and intact virions. Counter-immunoelectrophoresis (CIE) and microgel double diffusion were routinely used for detection of hexons, at different stages of purification. It was found that the CIE test could be used to demonstrate the presence and location of the antigen at low concentrations in a short period of time. Specific precipitates were evident after 1.5 h by Coomassie brilliant R blue staining (Fig. 14). Microgel double diffusion was also used for detection of group-specific antigen with fractions obtained from rate zonal centrifugation and DEAE-cellulose chromatography. After incubation of gels for 24 h at room temperature, the precipitation lines were revealed as sharp and distinct lines between the antigen and antibody wells (Fig. 15).

The results from both assay systems showed:

a) CIE can be used as a qualitative, rapid and reliable technique for detection of group-specific as well as type-specific determinants with EAdV soluble components (Fig.14)

b) Microgel double diffusion can also be employed as a simple and reliable assay (in parallel to CIE test) by using

Figure 10 (Top)

DEAE-Cellulose Chromatography elution profile of EAdV004 hexon Component

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Soluble Component - derived hexon was first separated in 5-20% sucrose linear gradients as described in Materials and Methods. Hexon positive fractions were pooled, concentrated with PEG-6000 and dialyzed versus Tris-HCl, pH 8.4 overnight. An aliquot was layered on top of the column and eluted with a linear gradient of 0-0.8M NaCl.

Symbols:

● Optical Density

+Intensity of CIE Precipitation line with heterotypic antiserum. The intensity was measured in comparison with the homotypic antiserum reaction.

Figure 11 (Bottom)

DEAE-Cellulose Chromatography elution profile of EAdV020 hexon Component

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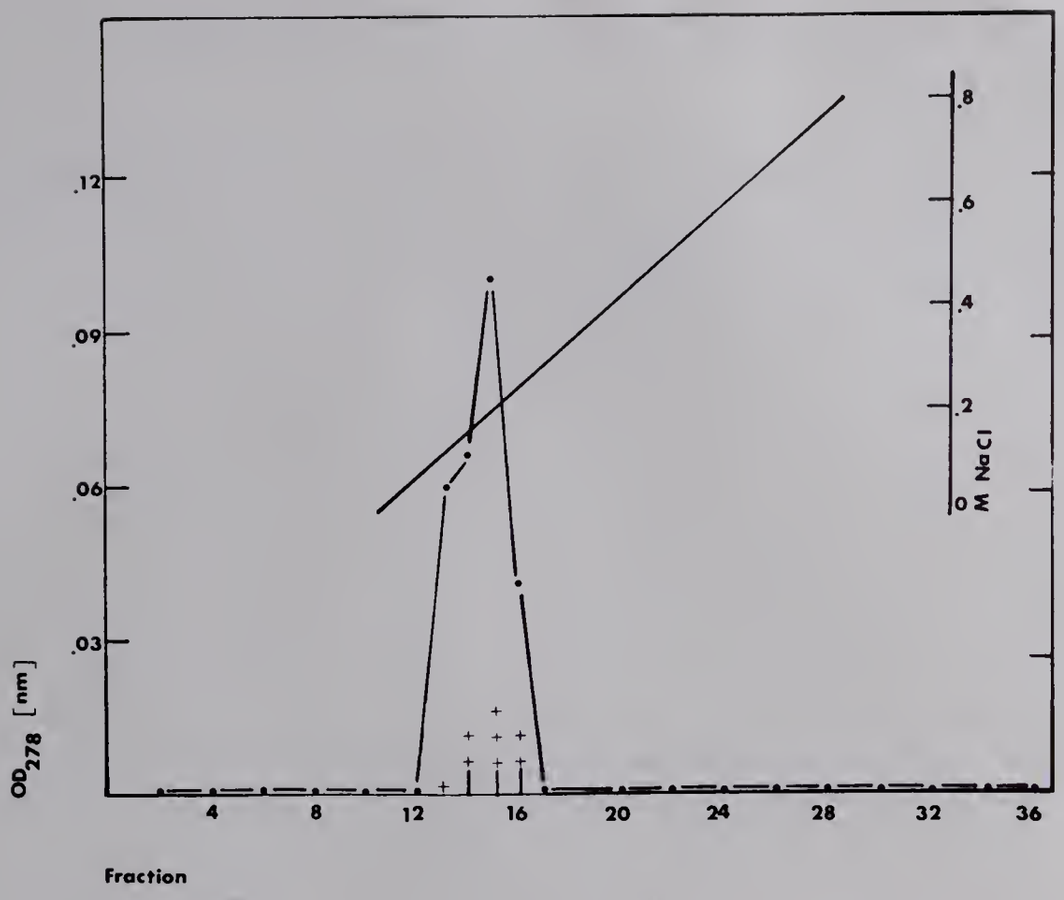
The experiment was performed as described in Fig. 10.


.12
.09
.06
.03
OD_{278} [nm]
4
8
12
16
20
24
28
32
36
Fraction
.8
.6
.4
.2
0
M NaCl

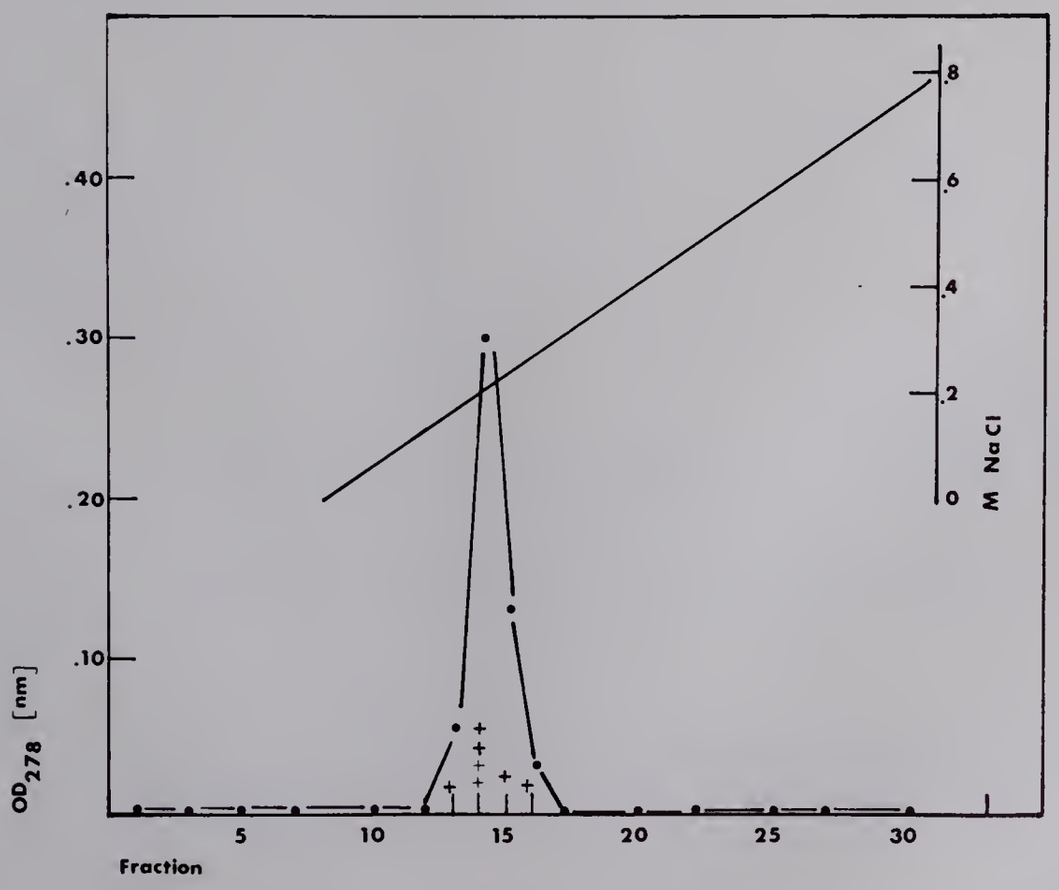

.40
.30
.20
.10
OD_{278} [nm]
5
10
15
20
25
30
Fraction
.8
.6
.4
.2
0
M NaCl

Figure 12 (Top)

DEAE-cellulose chromatography elution profile of EAdV008 hexon component

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The experiment was performed as described in Fig. 10.

Figure 13 (Bottom)

DEAE-cellulose chromatography elution profile of EAdV-B hexon component

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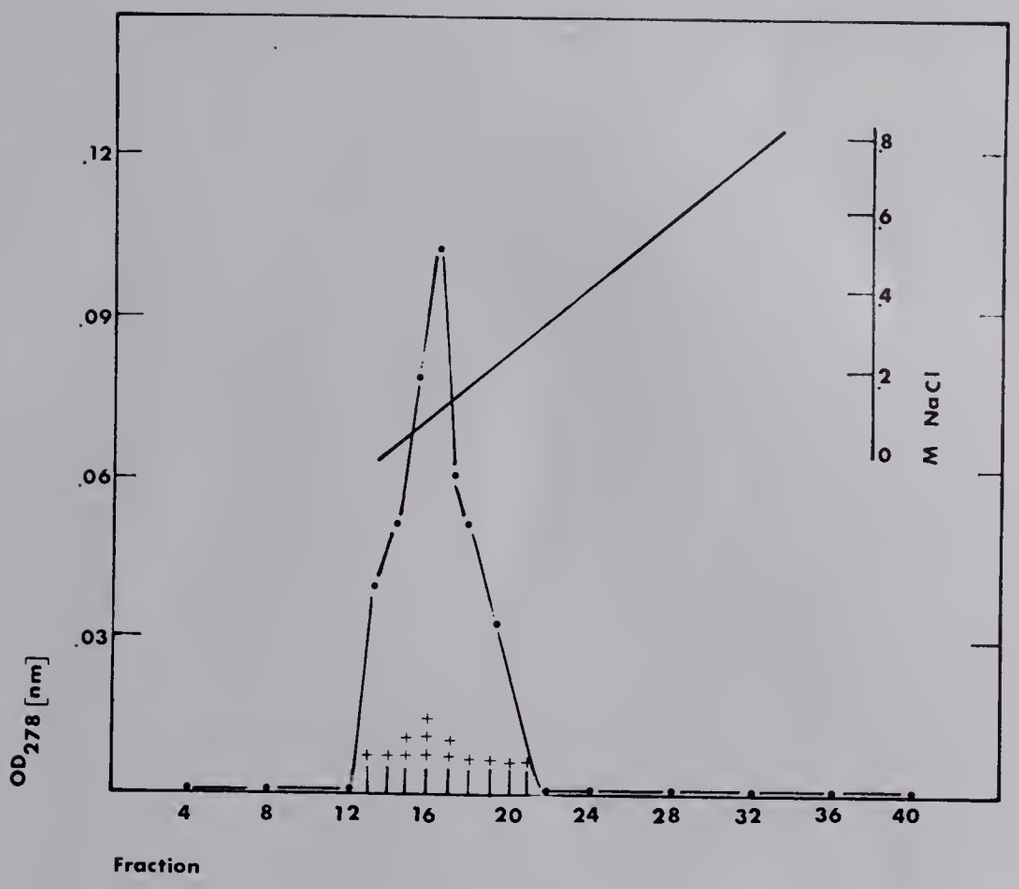
The experiment was performed as described in Fig. 10.


.12
.09
.06
.03
OD_{278} [nm]
4
8
12
16
20
24
28
32
36
40
Fraction
.8
.6
.4
.2
0
M NaCl

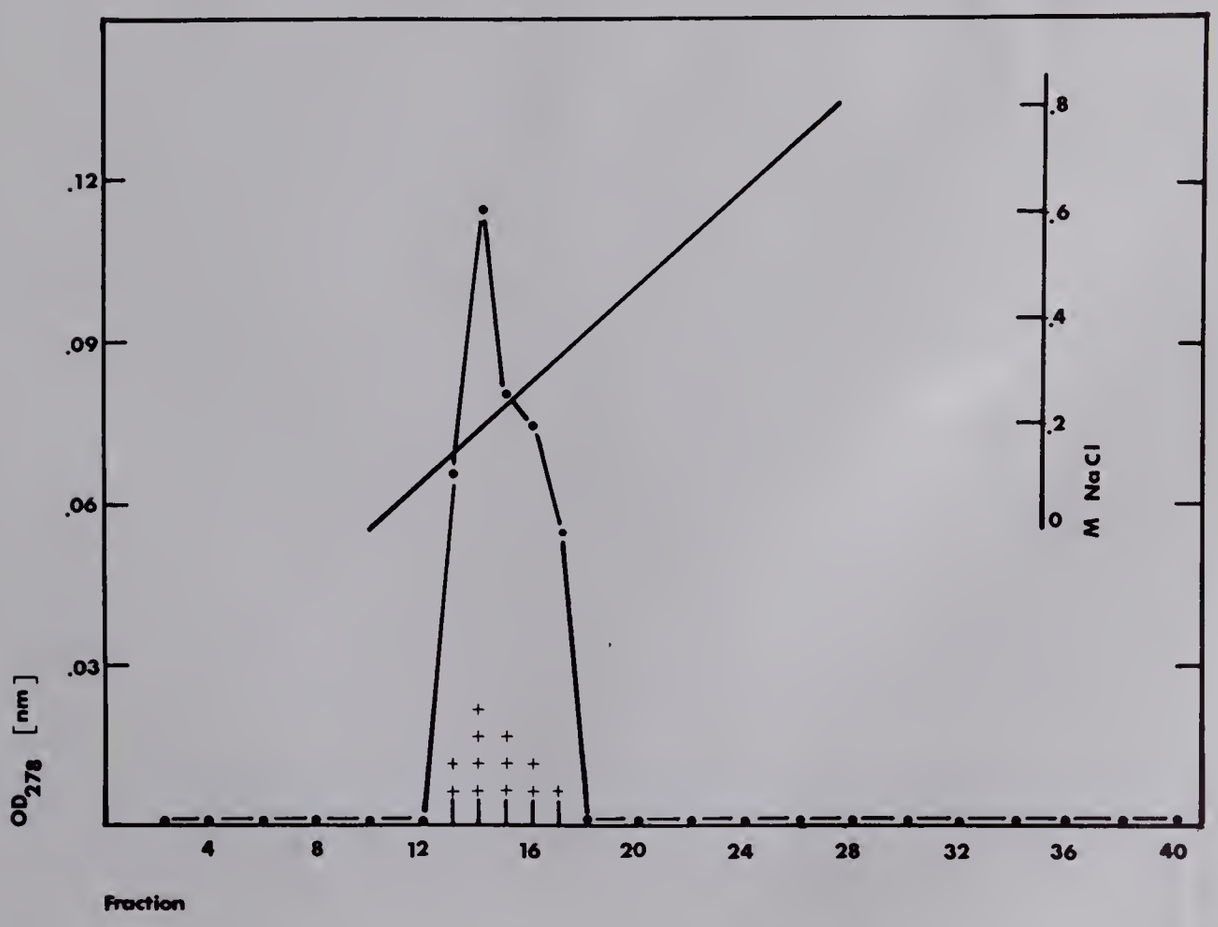

.12
.09
.06
.03
OD_{278} [nm]
4
8
12
16
20
24
28
32
36
40
Fraction
.8
.6
.4
.2
0
M NaCl

Figure 14 (Top)

Counterimmunoelectrophoresis of fractions obtained from DEAE-Cellulose Chromatography of EAdV-B

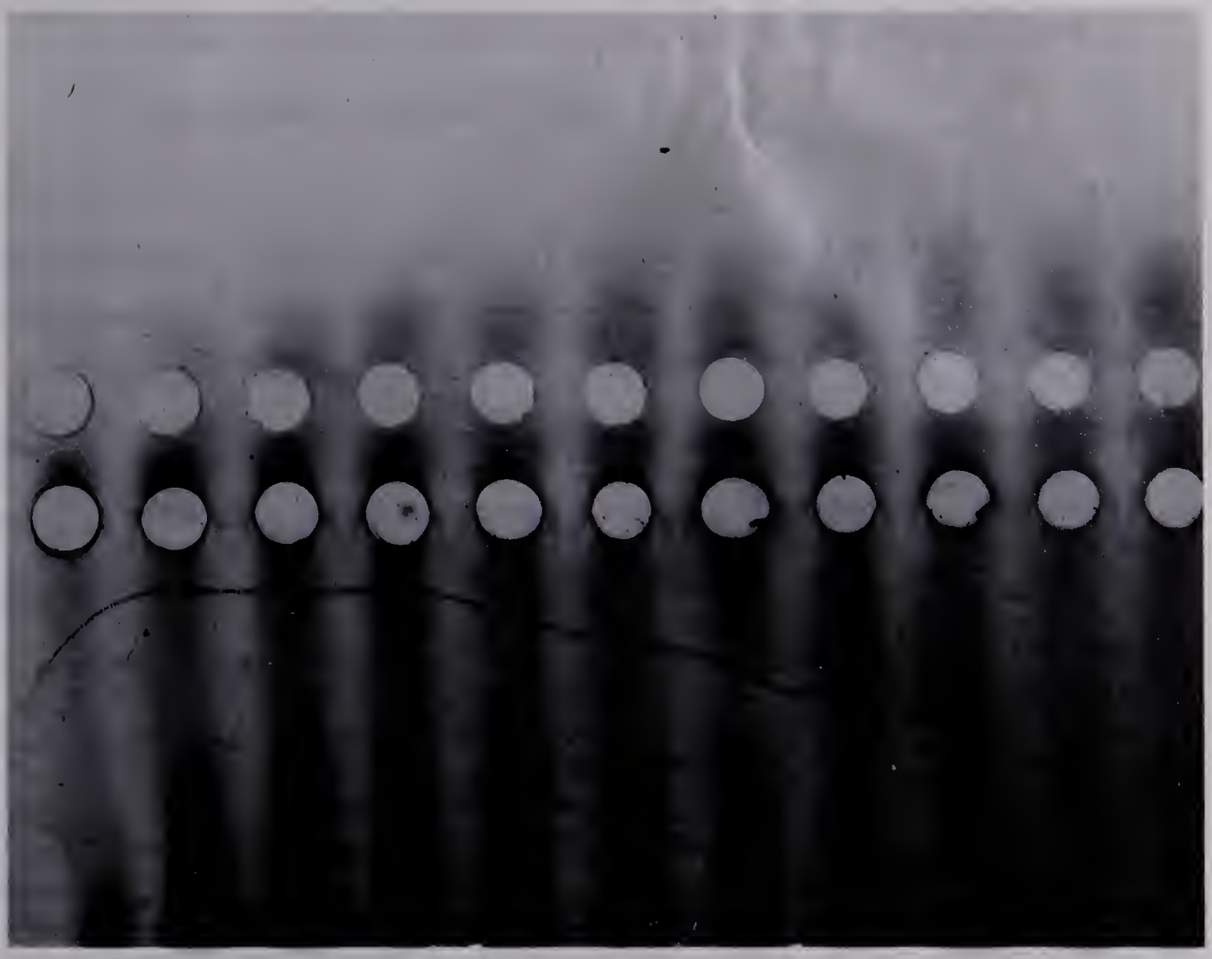
The plates for agar gel immunoelectrophoresis were prepared as described in Materials and Methods. 10 ul of each fraction eluted from the DEAE-cellulose column were added to each of the lower wells and 10 ul of heterotypic (against HAd-3) antisera added to the upper wells. CIE was performed in a Bio-Rad immunoelectrophoresis apparatus with 12 mA constant current for 1.5 h. The plates were then pressed, dried and stained with 2% Coomassie brilliant blue R for 10 min. The precipitation lines indicate the presence of the hexon component in fractions.

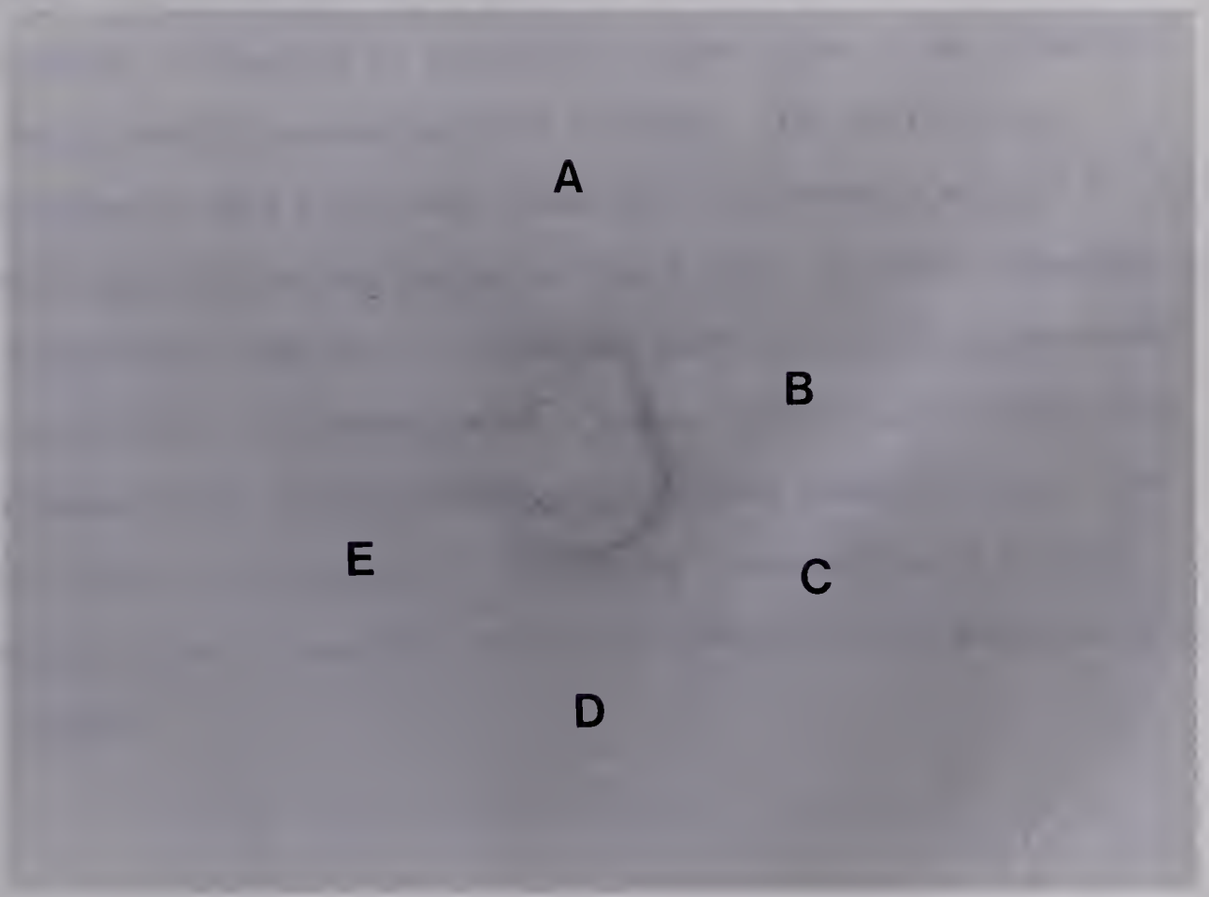
Figure 15 (Bottom)

Micro gel double diffusion of fractions obtained from DEAE-Cellulose Chromatography of EAdV-B

The slides for micro gel double diffusion were prepared as described in Materials and Methods.

20 ul of heterotypic (against HAd-3) antisera were added to the center well and 20 ul of the same fractions as in Fig. 14 were added to the wells designated A, B, C, D, E. Notice the precipitation lines with hexon positive fractions.




A
B
E
C
D

antisera against purified components of homotypic and heterotypic antigens. (Fig. 15)

12. Endonuclease activity with EAdV virion-derived pentons

An attempt was made to detect endonuclease activity in association with EAdV virion-derived pentons, as described in Materials and Methods. The results from this experiment are shown in Figure 16. The endonuclease assay was performed at two different pH's (7.4 and 4.5) and over a prolonged time interval. Very high endonuclease activity was detected with fresh preparations of virion-derived pentons at pH 7.4 after 60 min incubation with PM2 DNA at 37°C (10.5 unit increase in the fluorescence enhancement of ethidium bromide bound to duplex DNA). When this sample was heated a 38.5 unit decrease in the fluorescence was obtained, indicative of a helix-coil transition of the substrate DNA, facilitated by endonucleolytic cleavage. The activity of endonuclease at pH 4.5 was much lower than its activity at pH 7.4. The enzymatic activity in the sample decreased after ten days incubation at room temperature, indicative of the low stability of the enzyme under these conditions, in comparison with penton-derived endonuclease from human adenoviruses (Tsang and Marusyk, personal communication). The results from this experiment indicated the introduction of an endonucleolytic nick in substrate PM2 DNA by penton-derived materials from EAdV virions.

Figure 16

Fluorimetric assay of EAdV penton-associated endonuclease activity

---

Virion-derived pentons were prepared as described in Materials and Methods. The enzyme reaction mixture consisted of 0.01M Tris-HCl pH 7.4 or Citrate buffer pH 4.5 plus 0.1M NaCl, 0.002M $MgCl_2$ and 5 ug of PM_2 DNA and 10 ul of virion derived component suspension. Aliquots of 10 ul from the reaction mixture were added to 2 ml of ethidium bromide solutions (0.02M K_3P_i, pH 12, 0.002M EDTA, 0.5 ug of ethidium bromide per ml). The fluorescence was determined relative to a blank, with excitation taking place at 525 nm and emission at 600 nm in a Turner 430 spectrofluorimeter. The standard solution consisted of 25 ul of calf thymus DNA in 2 ml of ethidium bromide.

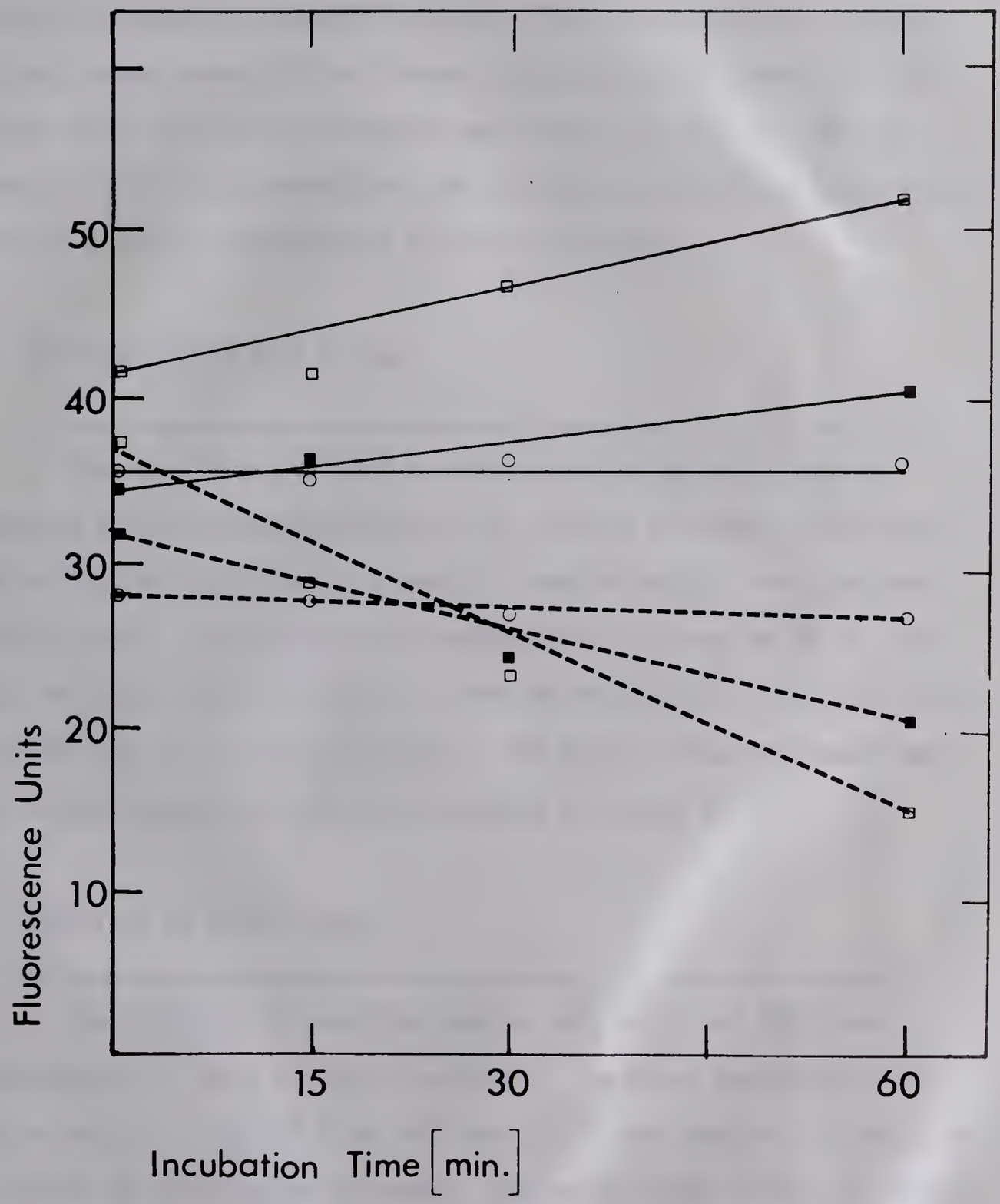
Symbols:

○ Blank

□ Sample in Tris-HCl, pH 7.4

■ Sample in Citrate buffer, pH 4.5

-□- -■- Shows samples after heating (98°C for 2 min)


50
40
30
20
10
Fluorescence Units
15
30
60
Incubation Time [min.]

13. Thermal denaturation of EAdV DNA

Various methods of DNA extraction (Pronase + SDS digestion followed by phenol or chloroform-butanol extraction) repeatedly failed to yield enough viral DNA for thermal denaturation measurements. Four to five ug/ml of DNA were extracted and found to be insufficient for detection of thermal transition. An ethidium bromide fluorescence assay revealed extensive denaturation of the purified DNA.

14. pH Stability of EAdV Virion

The sensitivity of EAdV to differences in pH was tested as described in Materials and Methods. The results in Figure 17 show that EAdV was not stable at pH 2, 3, and 11. The HA activity was completely destroyed after 1 h incubation of virion-buffer mixture at 23^{o} C. The virus was more stable at pH 4, 5, 6 than at pH 8 and 9; (4 fold decrease in initial HA activity was observed). The results from this experiment were in good agreement with those reported by Harden (1974).

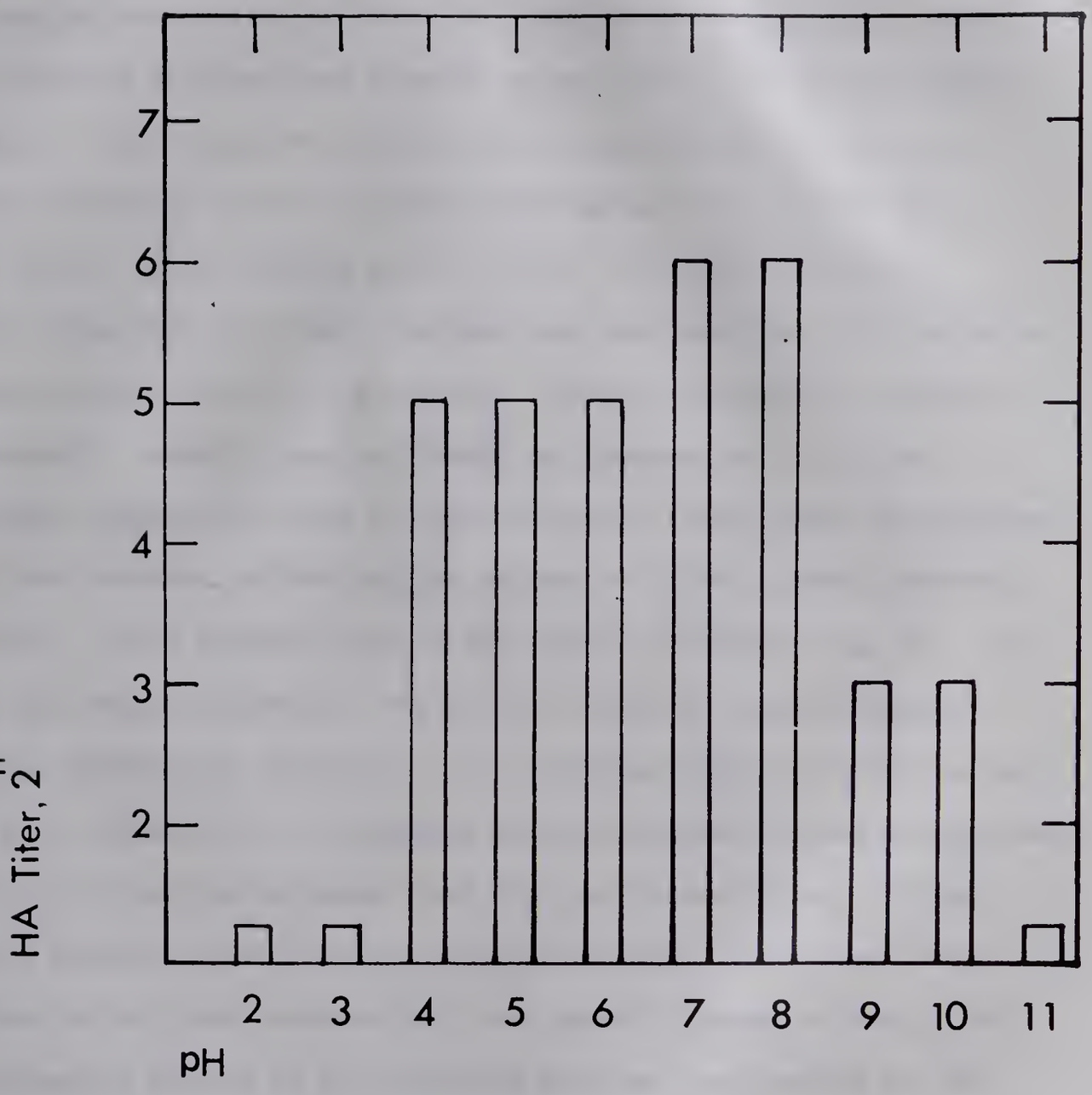
15. Stability of EAdV in CsCl

The stability of purified EAdV in CsCl at 4^{o} and 23^{o} C was determined by HA assay as described above. The virus was found to be stable both at 4^{o} and 23^{o} C in CsCl over a six week period. No decrease in initial HA activity was observed. The results were similar to those for human adenoviruses, which indicates the high degree of structural stability of this group of viruses in CsCl over a prolonged time (Marusyk, personal communication).

Figure 17

Effect of pH on the stability of EAdV

Two ml of purified virus suspension in PBS (initial HA titer 128) was dispensed in dialysis tubing and dialyzed versus 100 ml of buffer solution (pH2 to 11) for 1 h at room temperature. The virus-buffer mixtures were then dialysed against neutralizing buffer overnight. Two-fold dilutions of each sample were prepared and hemagglutination titers determined.


HA Titer, 2^n
7
6
5
4
3
2
2
3
4
5
6
7
8
9
10
11
pH

16. The SDS-polyacrylamide gel electrophoresis of purified EAdV Virion

Polyacrylamide gel electrophoresis was employed to analyze the polypeptide composition of four EAdV isolates at the molecular level. All four EAdV isolates were purified twice by CsCl equilibrium centrifugation. HAd2 virus was purified in the same manner and used as a known polypeptide chain molecular weight marker (Philipson et al., 1975: Weber, 1976; Anderson et al., 1973). The results given in Table V show that 12-14 major polypeptides were resolved, with molecular weights ranging from 9,500 to 145,000. (MW for polypeptide 1 was not determined). Results were consistent and reproducible in several different preparations with all EAdV isolates. Three minor polypeptides were also observed, with molecular weights of 28,500 (EAdV-B, EAdV004, EAdV020), 53,500 (EAdV004) and 78,500 (EAdV-B, EAdV020) (Fig. 18). For molecular weight estimation, the distance moved by each polypeptide band was measured by micrometer. All molecular weight calculations were done with reference to the accepted molecular weight of HAd2 polypeptides. The results from two different PAGE slab gels revealed that the polypeptide migration profile and calculated molecular weights was very similar for all EAdV isolates with only minor differences being noted. Densitometric tracing of the PAGE slab gels was also carried out for better determination of the relative mobilities of the resolved polypeptides.

The results of densitometric tracing (Fig. 19), detected 10 major polypeptides, designated VP1 to VP10.

Table V. Molecular weight of resolved equine adenoviruses polypeptides in discontinuous SDS-polyacrylamide gel electrophoresis

	Adenovirus			
Polypeptides	EAdV008	EAdV-B	EAdV004	EAdV020
1	-*	-	-	-
2	145**	145	145	147
3	131	128.5	129.5	129
4	100	99.5	100.5	94
5	98	95.5	98	81
6	89	87	90	(78.5)
7	67	(78.5)	67	66.5
8	64	66	63	64
9	61.5	62	61	(28.5)
10	21.5	58.5	(53.5)	20.5
11	11.5	(28.5)	(28.5)	11
12	9.5	20.5	20.5	9.5
13		11	11	
14		9.5	10	

* Molecular weight was not determined

** All values represent MW X 10^{-3} and are rounded to nearest five hundred for each polypeptide band.

Values in the bracket show minor polypeptides MW.

Figure 18

SDS-polyacrylamide gel electrophoresis of EAdV polypeptides

Four equine adenovirus isolates were purified by CsCl isopycnic centrifugation. Each sample was prepared for PAGE as described in Materials and Methods.

Slot designation	virus isolate in slot
A	purified HAd2 virions (marker)
B	purified EAdV004
C	purified EAdV008
D	purified EAdV020
E	purified EAdV-Briarwood
F	purified Had2 virions (marker)

C	same as above (notice the presence of the 1½ major polypeptides designated VP_1 to VP_{12})
D	same as above (the arrow indicates the presence of the polypeptide band missing in D)

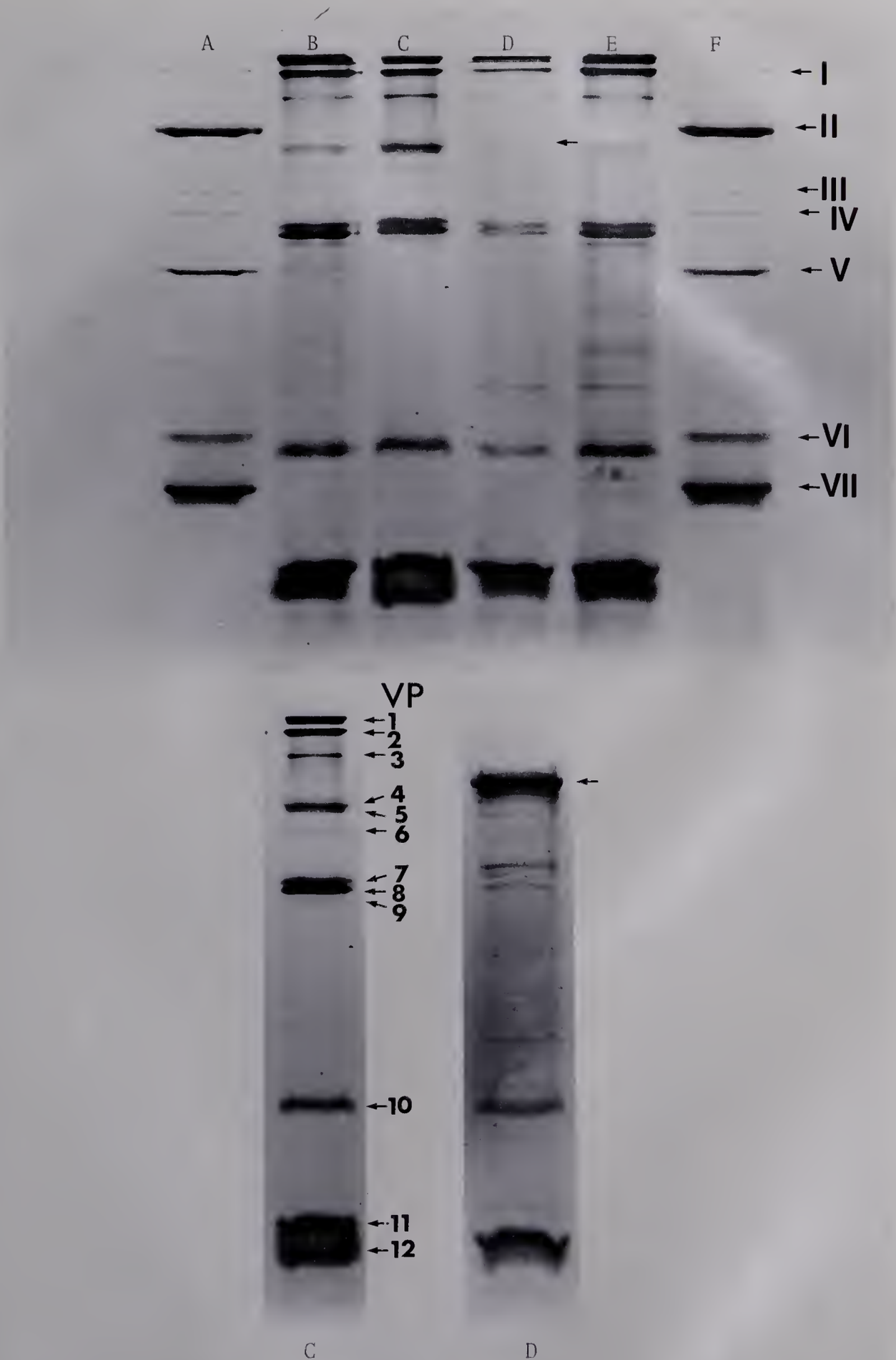

A
B
C
D
E
F
I
II
III
IV
V
VI
VII
VP
1
2
3
4
5
6
7
8
9
10
11
12
C
D

Figure 19

Densitometric tracings of four EAdV polypeptides separated by PAGE

The stained polypeptides from PAGE (Fig. 18) were scanned at 620 nm in a Gilford spectrophotometer 240 as described in Materials and Methods.

---

a. EAdV008 with designated polypeptides (VP_1-VP_{10})*

b. EAdV004

c. EAdV020

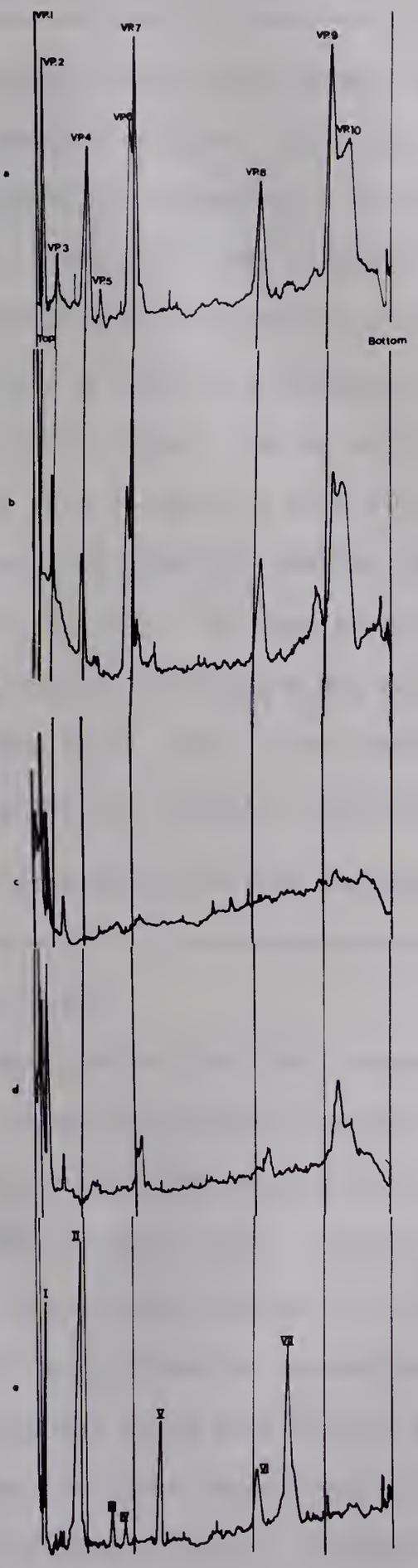
d. EAdV-B

e. HAd-2 with accepted polypeptide designations (I-VII)

---

* Assigned polypeptide numbers correspond to those in Fig. 18 and Table V as follows:

VP_1	(corresponds to polypeptide I)
VP_2	(145K)
VP_3	(131K)
VP_4	(100K)
VP_5	(98K)
VP_6	(67K)
VP_7	(64K)
VP_8	(21.5K)
VP_9	(11.5K)
VP_{10}	(9.5K)


VP.1
VP.2
VP.3
VP4
VP.5
VP.7
VP.8
VP.9
VP.10
a
Top
Bottom
b
c
d
e
I
II
V
VI
VII

17. Buoyant density of EAdV isolates in CsCl

The buoyant densities of the four purified EAdV were determined in CsCl after isopycnic banding of virus. The virus formed a sharp narrow band in CsCl following centrifugation. The density of the solution at the position of the virus band (effective density) was calculated by careful measurement of the weight of each fraction. In order that measurement error be kept to a minimum, all measurements were done using the same 100 ul pipet. The HA activity and OD_{260} peaks of the four EAdV isolates were observed to coincide at densities 1.345, 1.349, 1.344, 1.344 g/ml (EAdV020, EAdV004, EAdVB, EAdV008) respectively (Fig. 20, 21, 22, 23). The results were in good agreement with the value reported by Dutta (1975), but not to those obtained by Harden (1974) and Ardans et al. (1973), who reported buoyant densities of 1.33 and 1.31 g/ml for two EAdV isolates respectively.

18. Electron microscopy observation of EAdV isolates.

a) Morphology and fiber length

Morphological comparison of four EAdV isolates was performed by electron microscopy, taking advantage of the fact that such information (i.e. length of fiber) can be used for serotyping of adenoviruses (Norrby, 1969; Marusyk, 1972). Typical adenovirus morphology was observed in all EAdV isolates (Fig. 24, 25). It was shown that the four EAdV isolates have an unenveloped icosahedral symmetry with 6 solid capsomers along each edge of a triangular facet. The presence of six 50 nm long fiber projections in EAdV004 and EAdV020 isolates was of special interest (Fig.26). Attempts to visualize the fiber

Figure 20 (Top)

CsCl isopycnic centrifugation of purified EAdV020

Virus from CsCl step-gradient centrifugation (as described in Materials and Methods) was layered on top of CsCl with initial density of 1.399 g/ml and centrifuged to equilibrium for 24 h at 100,000 xg in a SW50 rotor. Fractions were collected and the buoyant density and hemagglutination titer of each fraction was determined.

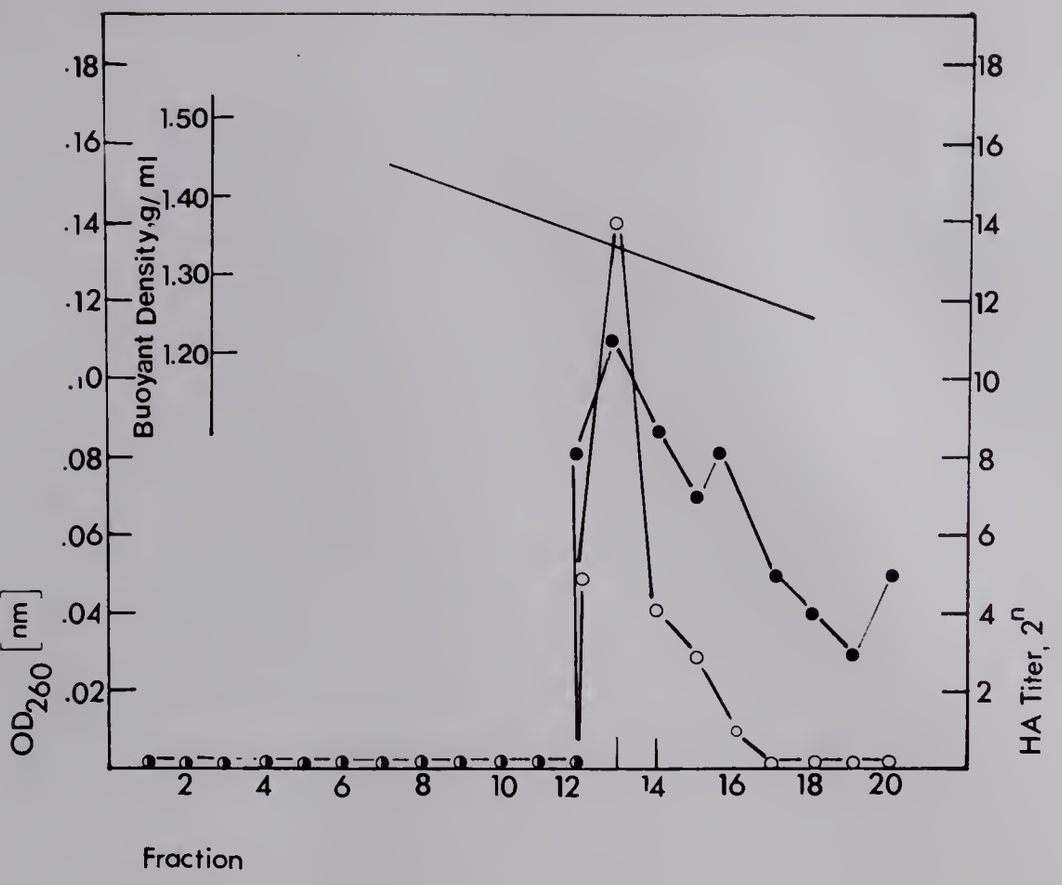
Symbols: o optical density

● HA titer, 2^n

Figure 21 (Bottom)

CsCl isopycnic centrifugation of purified EAdV004

The experiment was performed as described in Fig. 20


Buoyant Density, g/ml
1.50
1.40
1.30
1.20
OD_{260} [nm]
.18
.16
.14
.12
.10
.08
.06
.04
.02
HA Titer, 2^n
18
16
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20
Fraction

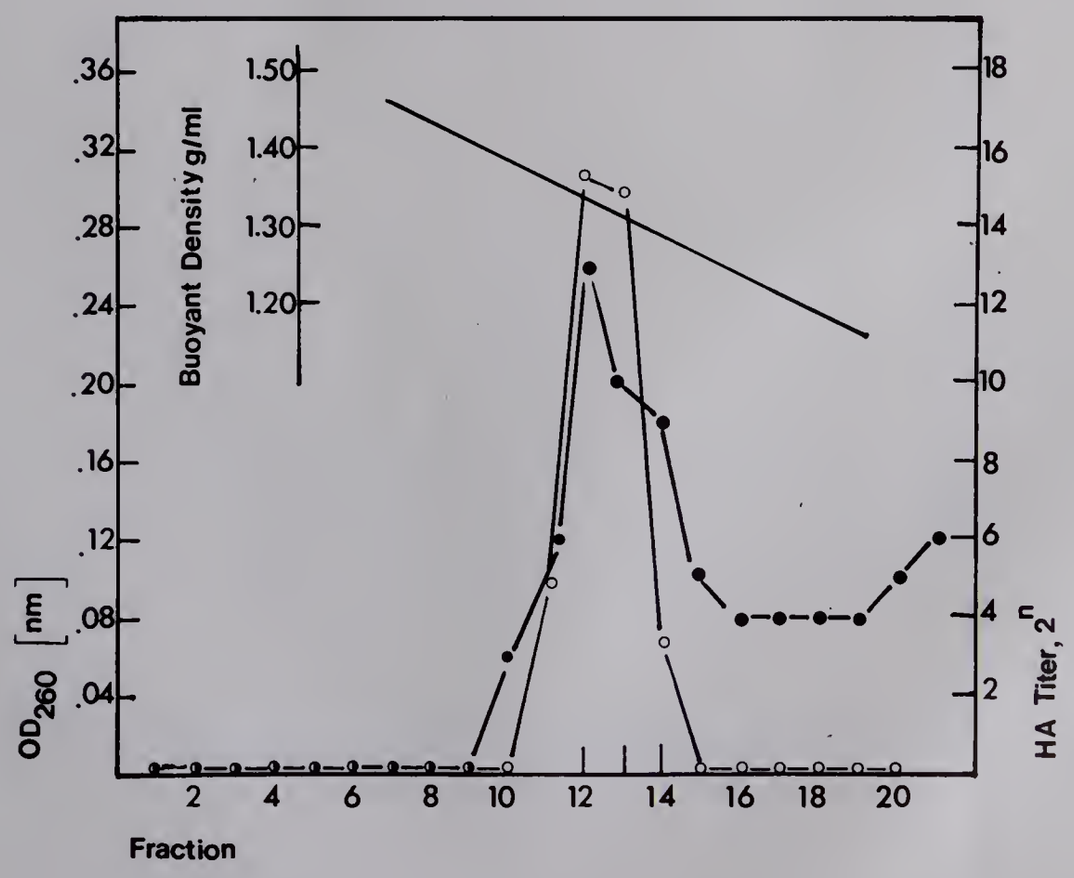

Buoyant Density g/ml
1.50
1.40
1.30
1.20
OD_{260} [nm]
.36
.32
.28
.24
.20
.16
.12
.08
.04
HA Titer, 2^n
18
16
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20
Fraction

Figure 22 (Top)

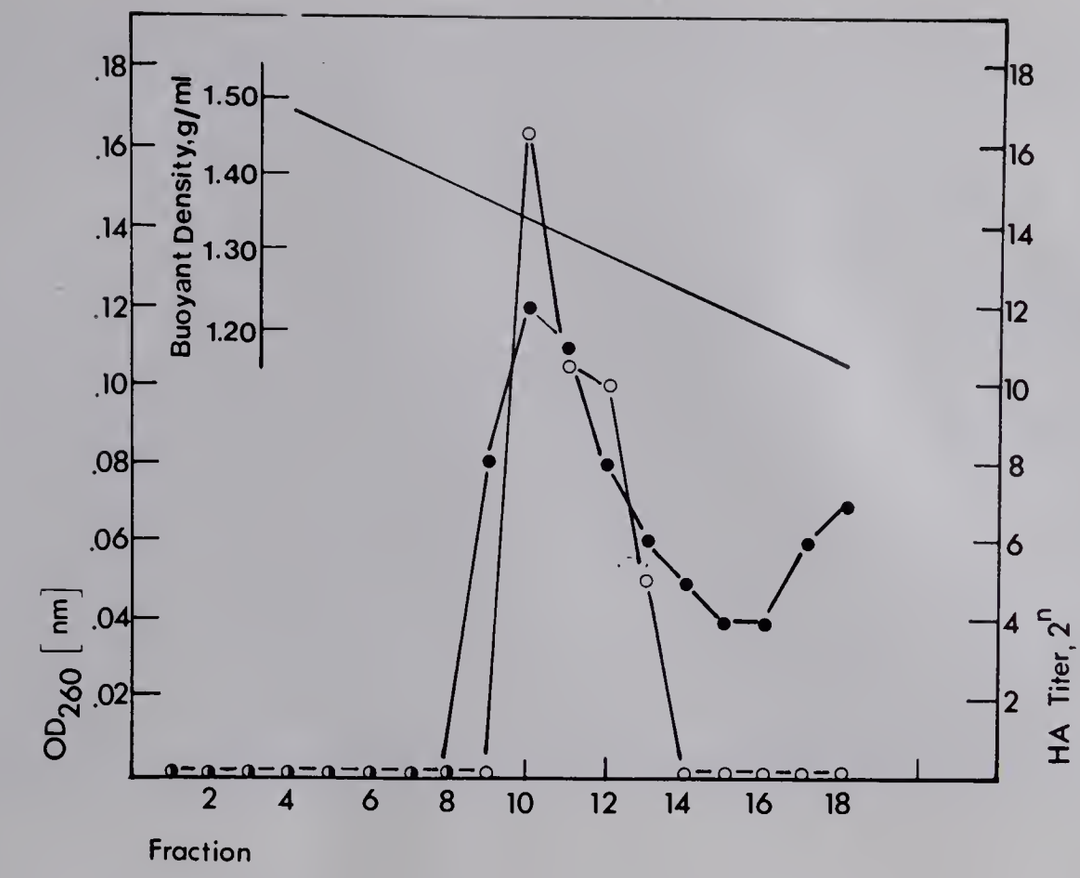
CsCl isopycnic centrifugation of purified EAdV-Briarwood

The experiment was performed as described in Fig. 20.

Figure 23 (Bottom)

CsCl isopycnic centrifugation of purified EAdV008

The experiment was performed as described in Fig. 20.


Buoyant Density,g/ml
1.50
1.40
1.30
1.20
OD260 [nm]
.18
.16
.14
.12
.10
.08
.06
.04
.02
HA Titer, 2n
18
16
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18
Fraction

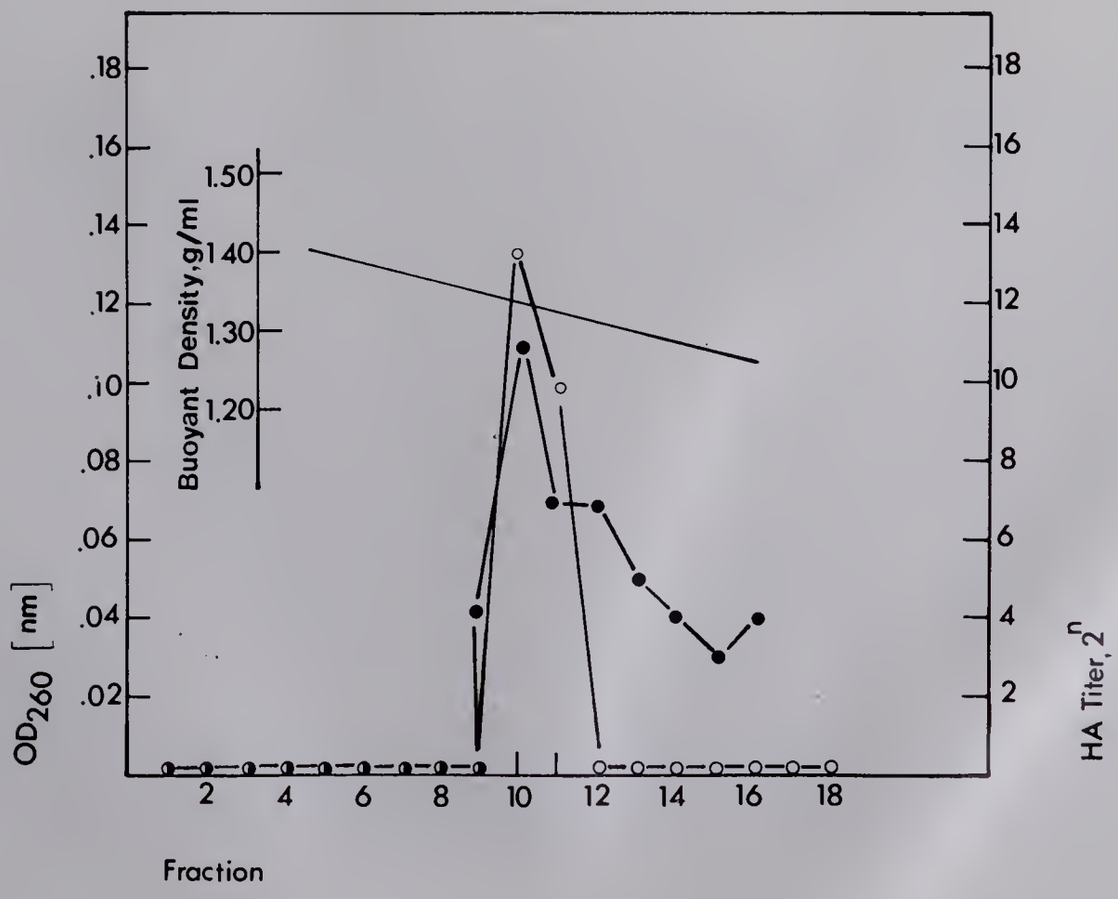

Buoyant Density,g/ml
1.50
1.40
1.30
1.20
OD260 [nm]
.18
.16
.14
.12
.10
.08
.06
.04
.02
HA Titer, 2n
18
16
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18
Fraction

Figure 24

Electron photomicrograph of purified EAdV-B

The virus was purified by two cycles of CsCl centrifugation and stained with 3% sodium silicotungstate, pH 6.8. Notice the cubic symmetry of the virus and the presence of an empty particle (arrow).

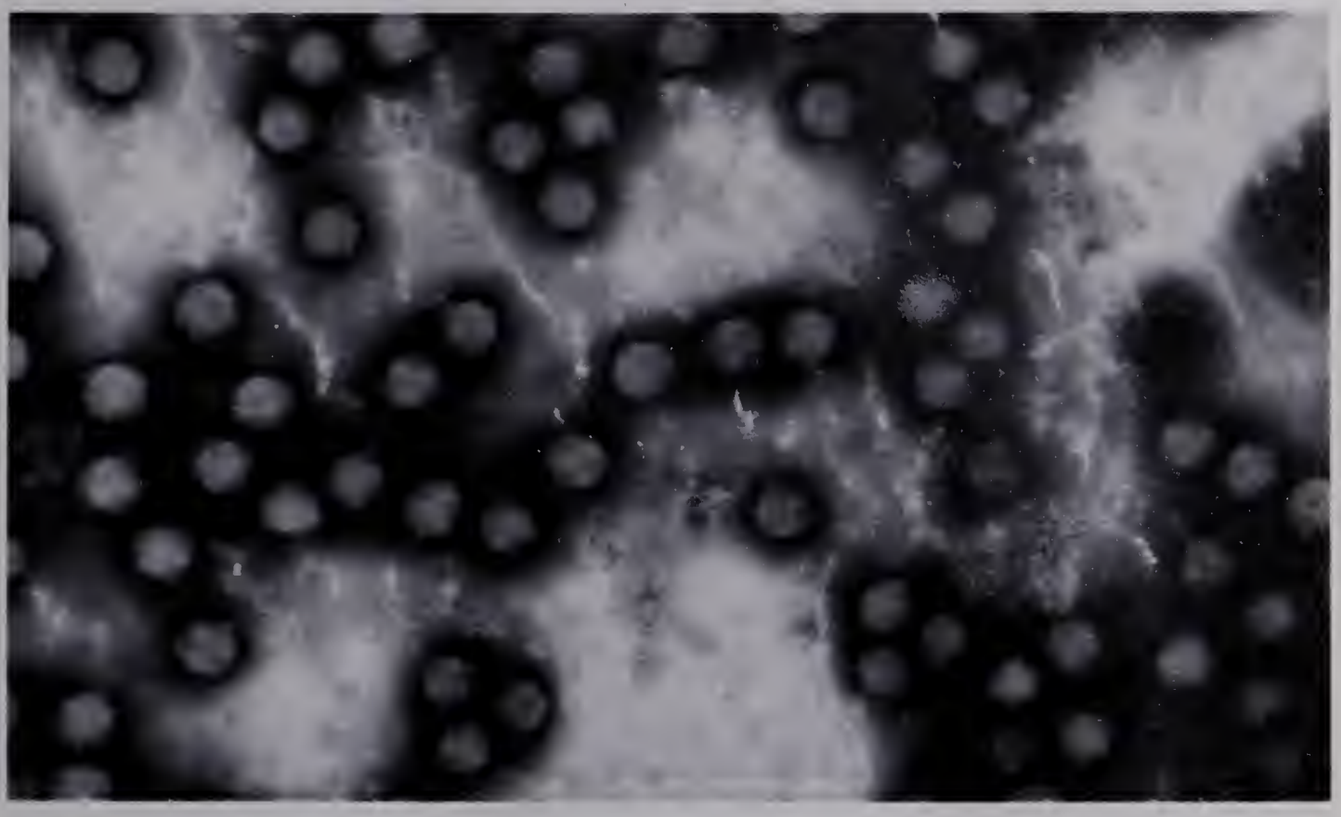
Magnification: X 73,600

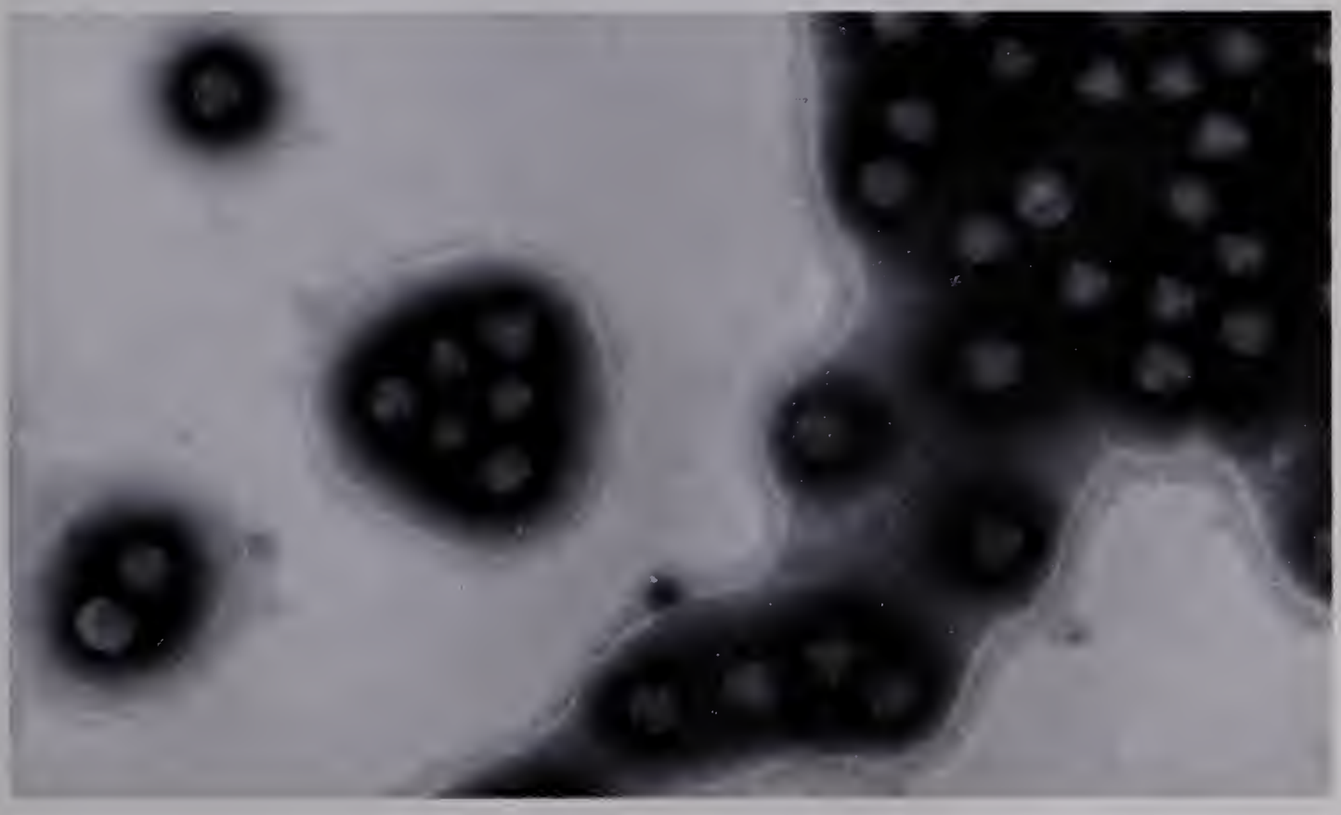
Figure 25

Electron photomicrograph of purified EAdV008

The experiment was performed as described in Fig. 24. Notice the similarity in morphology of the two isolates.

Magnification: X 73,600





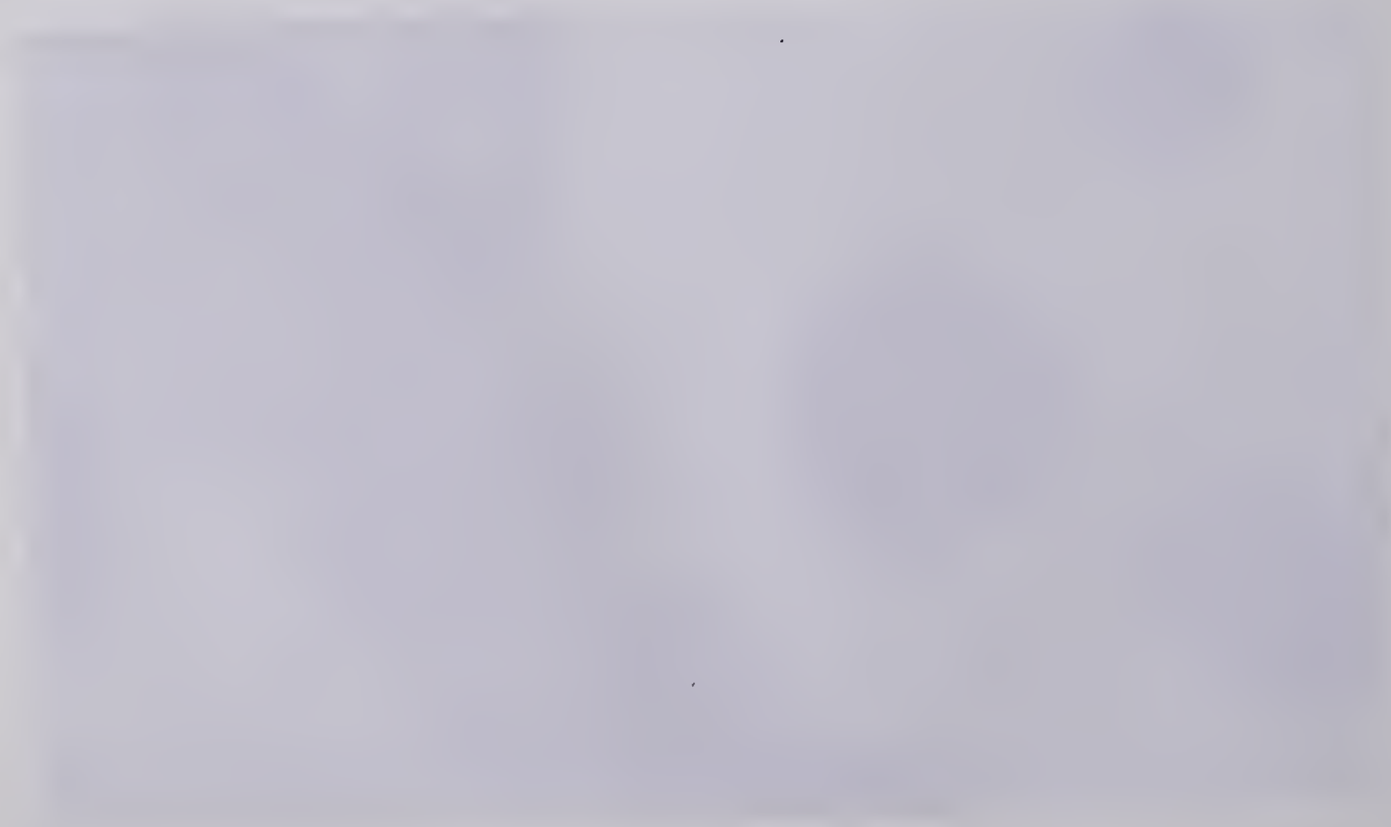
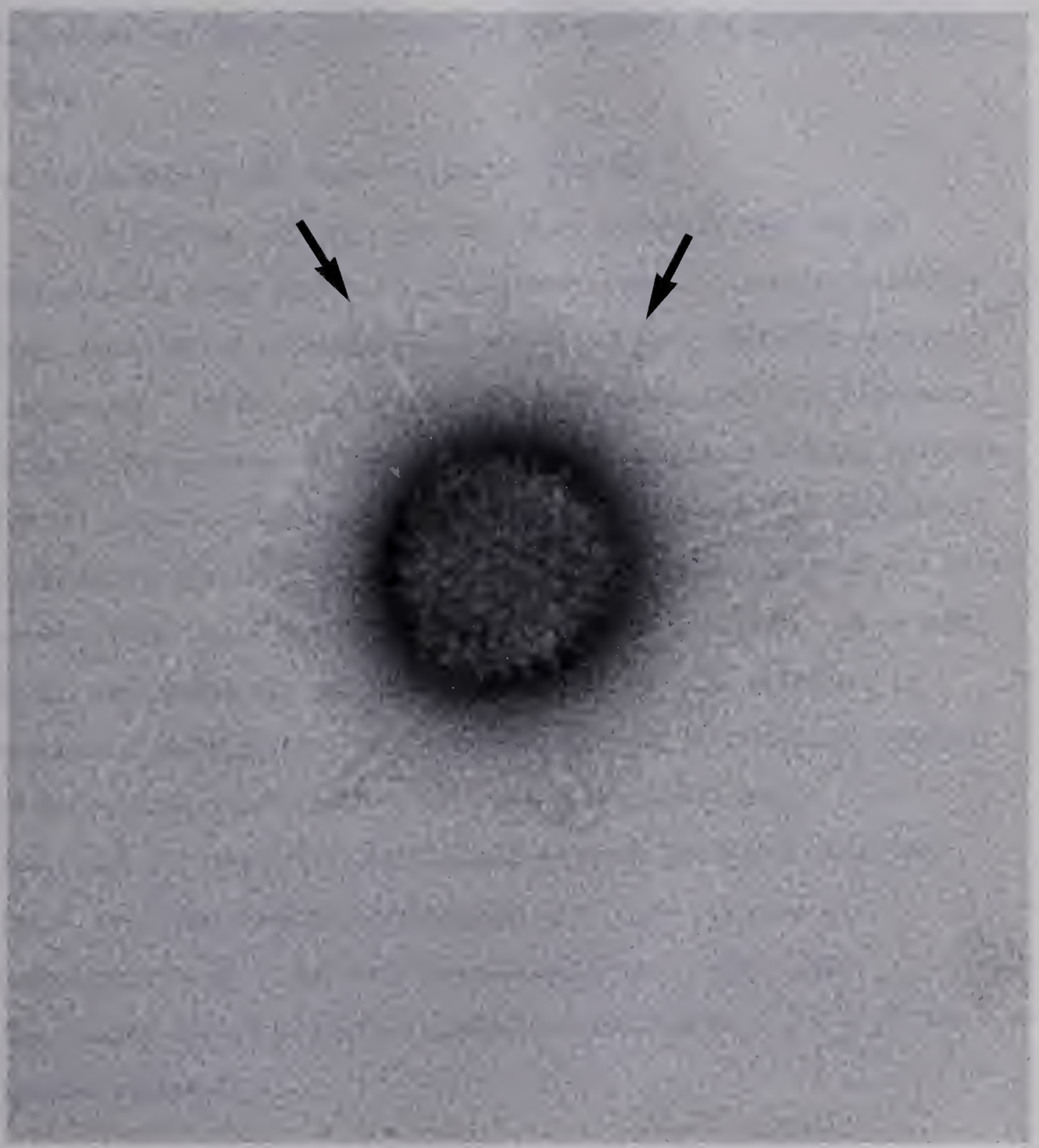


Figure 26

Electron photomicrograph of purified EAdV020

The virus was purified by two cycles of CsCl centrifugation and stained with 3% sodium silicotungstate, pH 6.8. Notice the presence of six fiber projections (arrows) with 50 nm length. Six solid capsomers are also evident along each edge of a triangular facet.

Magnification: X 556,000



projections on EAdV008 and EAdVB virions were unsuccessful. The length of fiber projections was determined on enlarged photographic images, assuming an average virion diameter of 80 nm. The length of the fiber projections was found to be identical for EAdV004 and EAdV020 isolates (50 nm).

b) Morphology and Molecular weight of EAdV DNA

Morphology and molecular weight of extracted EAdV DNA was determined by using purified virions from isopycnic centrifugation(Fig.27) Grids for electron microscopy were prepared by the diffusion method (Lang et al., 1964). The results (10 different electron micrographs) revealed the presence of intact duplex, full-length spread DNA molecules for all samples. No circular molecules were observed. In heavy metal shadowed DNA specimens the contour length and duplex structure was more evident (Fig. 28). Extensive fragmentation was also observed in some grids, indicating the susceptible nature of the long molecules to mechanical shear forces. The contour length of 10 individual samples of EAdV DNA was measured from enlarged photographic images which contained only one full-length spread molecule per field (Fig. 28). The molar linear density (daltons/cm) of T 7 DNA was used for calculation of MW of DNA from equine adenovirus. (Lang, 1970) The results showed an average molecular weight of 21 to 22 x 10^6 for samples examined.

Figure 27

Electron photomicrograph of EAdV DNA

Purified virus from isopycnic centrifugation was disrupted by addition of an equal volume of 6M guanidium hydrochloride. The grids containing DNA were stained with uranyl acetate. The molecular weight was calculated from the length of the molecule, as measured by map tracer device and from the molecular weight of T7 DNA.

Magnification: X 6,865

Figure 28

Electron photomicrograph of EAdV DNA

The viral DNA was prepared as described in Fig. 27, and shadowed with platinum -palladium at 15 degrees in a shadow-casting apparatus and examined in the EM.

Magnification: X 9,875

DISCUSSION

The results of various observations in this study have been employed in the classification of four equine adenovirus isolates at the molecular level.

Initial studies were undertaken to determine the efficiency of various cell culture systems for the study of EAdV isolates. The finding that equine primary kidney cells were the most efficient and practical host system for propagation, plaque formation and other routine experiments with the four EAdV isolates studied, suggested the use of such cells as the host system of choice. The virus yield obtained in the EPK cell system, as determined by infectivity tests and electron microscopic observations, was found to be unchanged relative to the multiplicity of infection used for all virus isolates. Feline primary kidney cells, the only other cell type giving high virus yield similar to that observed in EPK cells, was a suitable alternate cell system for propagation of EAdV. The propagation of EAdV in EDC and MDCK lines was found to be more efficient than virus yield obtained in rabbit primary kidney cells, VERO and BHK-21 cell lines, in terms of number of infectious particles obtained.

The propagation of EAdV in FPK cells and MDCK line (non-primate origin cell cultures) was found to be interesting with regard to the availability of EAdV specific receptors on the surface of these cells. Animal viruses are generally considered to be taken into cells after specific interaction of virus with a specific receptor site on the cell surface (Dales, 1965; Joklik, 1964; Philipson et al., 1968). The lack of growth of EAdV isolates in human cells is most probably the

result of absence of specific receptors in the particular cell line (diversity in cell receptor specificity).

Arginine has been reported to have an important role as a major adenovirus core constituent amino acid, or maturation factor, and also as an essential factor for transportation of synthesized proteins from the cytoplasm to nucleus (Everitt et al., 1971). The presence of supplemental levels of arginine in the maintenance medium in this study was also found to be helpful for enhancing the production of the virus.

Morphological alterations produced by an EAdV isolate in EPK cells at different time intervals PI (as analyzed by histological and EM observations) showed a sequential change in the amount of eosinophilic and basophilic materials in the nucleus of infected cells. These results clearly showed a direct involvement of the nucleus as the active site for synthesis and assembly of the EAdV isolates. Similar results have been reported by Wilk & Studdert (1973), Dutta (1975), and Shahrabadi et al. (1977).

When the morphology of plaques and plaquing efficiency of the different EAdV isolates were compared, no distinct differences were observed during the time course of plaque formation, and therefore no obvious strain differentiation was possible.

The method of choice for maximal recovery and purification of EAdV isolates from degenerating EPK cells was found to be sodium deoxycholate extraction, followed by two cycles of CsCl centrifugation. The results obtained in this study were essentially the same as those obtained with human Norrby (1966) and canine adenoviruses (Marusyk et al., 1970). The degree of purity of each virus isolate was monitored by measurement of the $A_{260/280}$ nm ratio and by electron

microscopy. The sodium deoxycholate extraction was especially efficient when compared with the fluorocarbon (Freon) extraction method and can, therefore, be suggested as a standard procedure for purification of this group of viruses. The one-step multiplication cycle of all adenoviruses has been reported to be essentially similar, especially in regard to time course, indicating the same possible sequence for synthesis and assembly of this group of viruses (Green et al., 1970; Philipson et al., 1973). When EPK cells were infected at high MOI and the lytic cycle of EAdV followed at 37° C, a synchronous two-step phase response was observed, as also reported by Harden (1974). A 16 h latent period was followed by another 16 h rise period. When mature virus particles were first detected at 16 h PI, they were mostly cell-associated, as expected because of the continuous intranuclear development of virus particles in infected cells.

The results of serological tests with soluble components, crude virus and specific antisera for serotyping of adenoviruses have long been a controversial issue. Purified hexon of certain human adenoviruses has, in some cases, been found to be non-immunogenic. Wadell (1970) and Pettersson (1971) have shown that only one sub-population of hexon purified by ion-exchange chromatography can induce the production of neutralizing antibody. Maizel et al. (1968) and Prage et al. (1970) have also postulated that hexons forming the complete virion structure are associated with a small extra polypeptide which is presumably responsible for production (enhancement?) of neutralizing antibody in animals. And finally, Stinski and Ginsberg (1975) have shown that isotopically-labeled surface polypeptides in unassembled purified hexons are different from those in intact capsids. The finding

that polypeptides exposed on capsid-incorporated hexons are not as readily exposed in unassembled purified hexon from cell homogenates (tertiary structure of the two classes of hexons), clearly indicates that the regions of structural similarity are critically located for each adenovirus serotype. The data suggest that, immunologically, the hexons in viral capsids react differently from unassembled hexons because the polypeptide chains are slightly different (configuration) in the two hexon forms, and therefore expose different regions of the protein to antibodies. However, the type-specific determinant may consist of variable regions in the polypeptide chain, and the group-specific determinant as a region with common primary amino acid sequences in all groups of adenoviruses except those of avian and bovine origin.

With the above discussion in mind, and little data available regarding serum-neutralization of EAdV isolates (Studdert, 1974), a careful cross-neutralization study with purified virions of four EAdV isolates and specific antibody was carried out. The results presented in this study have shown that no distinct antigenic diversity exists between the four EAdV isolates studied, as revealed by cross neutralization and hemagglutination-inhibition tests.

Neutralization-enhancement tests have been shown to detect non-critical sites on the surface of the virion (Norrby & Wadell, 1972; Wadell, 1972; Marusyk, 1972). Non-critical sites are sites to which antibodies attach without causing any detectable neutralization, and cannot, therefore, be detected in a non-sensitized assay system. The results of neutralization-enhancement tests in this study have not indicated the presence of such critical distinguishing sites on the

surface of the equine adenoviruses studied, thus confirming the previous serum neutralization results.

Marusyk (1972) reported the presence of shared inter-species determinants between human and canine adenoviruses, located on the penton base and proximal part of the fiber of these viruses as detected by hemagglutination-enhancement assays. The HE test can also detect the presence of monomeric structures in the soluble component population (penton and fiber by demonstration of incomplete HA). The observations from such tests with concentrated soluble components of EAdV isolates, using antiserum against HAd-6, failed to show either inter-species specificity or the presence of monomeric structural components. However, it can reasonably be assumed that the absence of such activities is probably the result of a low concentration of these monomeric structures in soluble component suspensions of EAdV isolates. Norrby (1971) and Marusyk (1972) have also reported the presence of non-virion associated components with complete HA activity in soluble component material of human, simian and canine adenoviruses. The results from such tests with highly concentrated soluble components of EAdV isolates from rate zonal centrifugation reveal the presence of a low concentration of oligomeric structures which are assumed to be penton dimers because of their relative sedimentation property. However, because of the low concentration of materials detected, the exact nature of the oligomeric structures was not determined.

Two-step purification of hexon of EAdV isolates, using rate zonal centrifugation and ion-exchange chromatography was found to be sufficient for obtaining this component in a relatively high state of purity, as revealed by immunoelectrophoresis and polyacrylamide gel

electrophoresis. Four-step purification of hexon of HAd2 using agarose chromatography, ion-exchange chromatography, preparative polyacrylamide gel electrophoresis and sucrose gradients with vast amounts of soluble components has been reported by Pettersson et al. (1967). When counter-immunoelectrophoresis and simple diffusion were employed for detection of hexon-associated group and type specific antigens, the results showed that both techniques can be employed, with a high degree of reliability and sensitivity, for detection of these specificities at different steps of purification.

The observation that elution profiles of four EAdV hexons from ion-exchange columns were very similar suggested that all four isolates carry the same relative net charge on their hexon and in regions of the polypeptide probably related to type-specific antigenic determinants. Stinski and Ginsberg (1975) have shown that isoelectric focusing values for polypeptides of hexon from HAd2, 5 (subgroup III) and type 3 (subgroup I), when prepared by treatment with cyanogen bromide and separated by isoelectric focusing in polyacrylamide gel, were similar for polypeptides originating from a common segment and completely distinct for those from variable segments of this component. The variability was shown to be far greater between type 2 and 3 hexons than between the hexons of type 2 and 5. The elution profile of soluble components from two canine adenoviruses was also shown to be markedly different in ion-exchange chromatography. These two adenoviruses have been shown to be distinct serotypes in hemagglutination-inhibition and serum-neutralization tests (Marusyk et al., 1970).

The presence of endonuclease-like activity in association with certain purified structural components of adenoviruses has been

postulated to be a common feature within the adenovirus group (Marusyk et al, 1975). No virion or component-associated endonuclease activity has been reported in association with purified virion-derived structural components of non-primate origin adenovirus as was shown in this study with purified pentons of EAdV. However, the nature of the endonucleolytic activity (double or single stranded scission; Burlingham et al., 1971; Marusyk et al., 1975) associated with EAdV on substract DNA was not pursued. The possible biological role of the endonucleolytic activity associated with this group of viruses remains to be determined. Recent studies have indicated that the activity may be a manifestation of a DNA-binding protein (Tsang and Marusyk, personal communication).

The transmission of equine adenoviruses in nature has been shown to be via a non-fecal route (MacChesney et al., 1970). This is in contrast to many of the human adenoviruses which may be transmitted primarily by the fecal route. Equine adenoviruses produce inclusions in epithelial cells of many tissues, including the urinary bladder, but not in the stomach or small and large intestine of the victims of the generalized adenovirus infection. The above observations are in agreement with results of pH stability experiments which have shown the EAdV is more sensitive to alkaline pH than acidic pH. Harden (1974) showed that an equine adenovirus isolate remained infectious after 24 h in acidic pH's. The EAdV was also found to be stable in 3 M CsCl at room temperature and 4° C (this study).

Polypeptide composition analysis by polyacrylamide SDS-gel electrophoresis has been used with great confidence (accuracy of at least $\pm$ 10%) for a wide variety of viruses and proteins (Weber and Osborn, 1969). Results of such experiments, as well as morphological

and biological characterization, can be adequately used for the study of diversity among adenovirus serotypes (Philipson et al., 1975; Marusyk and Cummings, in press). Electrophoretic analysis of the four purified equine adenovirus isolates on discontinuous polyacrylamide slab gels has shown the presence of 12 to 14 apparent constituent polypeptides ranging in size from 9,500 to 145,000 daltons. Certain polypeptides, not consistently found in all EAdV isolates (minor polypeptides), have molecular weight between 28,500 to 78,500. Three polypeptides with high molecular weight, 129,000 (VP2), 145,000 (VP3) and VP1 (MW not calculated) probably do not exist as single structural entities in the capsid of virus isolates, and are probably the result of aggregation of monomeric proteins. The possibility that minor polypeptides (observed) were, in fact, host protein contamination seems unlikely because of the extensive purification of each virus isolate prior to PAGE SDS-gel electrophoresis. However, such a possibility cannot be ruled out. A second possibility is that minor polypeptides may exist in different proportion in capsids of different isolates and the appearance of such in the PAGE SDS-gels may differ in each sample prepared. The nature of each polypeptide was not determined because of the difficulty in obtaining the same relative concentration of viral protein for each experiment.

When the polypeptide profiles in PAGE SDS-slab gels of the four EAdV isolates were compared, a high degree of similarity was observed in appearance and the calculated molecular weight for each polypeptide population. This indicated that the four EAdV isolates were similar when examined at the molecular level. The results obtained were in good agreement with those reported by Marusyk and Cummings (in press)

with another EAdV isolate. However, when the polypeptide profiles of the four EAdV isolates was compared with HAd-2, it was found that distinct differences exist in polypeptide migration of resolved peptides between the primate and non-primate adenoviruses. These differences clearly revealed the diversity of the two adenovirus sub-groups at the molecular level.

The assignment of identification numbers to the major polypeptides of EAdV isolates was done arbitrarily and with regard to their relationship to each other in the gel. The positive identification of each polypeptide population in terms of the existing single structural protein in capsids of this group of viruses was not pursued and remains to be determined. The relative mobilities of two polypeptides from the resolved EAdV isolates, VP4 and VP8 (MW 100,000 and 21,500); were found to be close to that of polypeptides II and VI of HAd-2 (MW 120,000 and 22,000, respectively). The relationship of polypeptides II and VI to the HAd-2 virion has been well established to be hexon (II) and ninemer (associated with groups of nine hexons), respectively.

The buoyant density of virus particles has been shown to depend primarily on their chemical composition and structure (Weigle et al., 1959; Sehgal et al., 1970; Antek et al., 1976). From the data obtained in this study, it was found that all four EAdV isolates are homogenous in regard to their nucleic acid and protein composition. The results of such experiments were in agreement with PAGE SDS-gel electrophoresis and clearly show a constant ratio of protein to nucleic acid for all EAdV isolates. Ardans et al. (1973) and Harden (1974) reported densities of 1.31 and 1.33 g/ml respectively for two equine adenovirus isolates. These values appear to be underestimations and might be the

result of different experimental conditions employed by the above workers. Dutta (1975) has reported a density of 1.34 g/ml for an EAdV isolate which is in agreement to the values obtained in this study. It should be emphasized that differences of 0.1 to 0.3 g/ml obtained by the above workers can be highly significant in terms of the protein and nucleic acid composition of virus isolates being studied, and in comparison to the values presented in this study.

Electron microscopic observations from this study have shown that all four EAdV isolates display cubic symmetry with six capsomers along the edge of each triangular facet. Using the formula $10\ (n-1)^2 + 2$ (Wildy and Watson, 1962), where 'n' is the number of capsomers along the edge of each triangular facet, a total of 252 capsomers was calculated for the EAdV isolates. This value has been well accepted for all adenoviruses, and has been previously reported for another EAdV isolate (Wilks et al., 1973). The presence of fiber projections on surface of EAdV isolates has not been reported previously. The results presented in this study have shown the presence of fiber projections measuring 50 nm long. Fiber structural components of such length were found to be very sensitive to mechanical forces, even after a gentle treatment for EM observations. Only a few virus particles were found with intact fibers on the virion in the samples prepared. This technical difficulty may have been the major reason for the failure of other workers to visualize fibers on virions of EAdV isolates.

Observations of the nucleic acid of equine adenovirus have shown that the viral genome exists as a linear duplex molecule, without segmentation or circular structures. The calculated size of $21 - 22 \times 10^6$ daltons was found to be in agreement to that obtained from

other adenoviruses, eg. HAd-2 - 23 x 10^6 daltons (Doerfler et al., 1970; Green et al., 1967; Van der Eb et al., 1969). A genome of this size can theoretically code for 25-50 average size polypeptides. Having in mind the accuracy of PAGE SDS-slab gels (±10%) and assuming that the polypeptides VP1 (145,000) and VP2 (129,000) from the PAGE analysis are the aggregate products of other structural proteins (as discussed above), and also taking into account two other virus specific polypeptides (72,000 and 41,000 daltons) which appear at an early stage of infection (8-10 h) in adenovirus-infected cells, the total molecular weight of all viral-related proteins detected in the PAGE SDS-gels is within 75,000 daltons of the lower theoretical limit (1.25 x 10^6 daltons).

On the basis of data accumulated in this study, it can be stated that there are no differences in the immunological, biophysical and biochemical properties of the four equine adenovirus isolates (EAdV004, EAdV020, EAdV-Briarwood, EAdV008) and it is suggested, therefore, that the EAdV isolates listed above be considered as a single antigenic serotype.

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